DOES NOT CIRCULATE

Volume 54 APRIL, 1954 Number 4

ENDOCRINOLOGY

UNIVERSITY OF MICHIGAN
APR 13 1954
HOSPITAL LIBRARY

ROSEMBERG, EUGENIA, JEROME CORNFIELD, ROBERT W. BATES AND EVELYN ANDERSON
Bioassay of Adrenal Steroids in Blood and Urine Based on Eosinophil Response: A Statistical Analysis 348

DAVIS, M. EDWARD AND ERNST JURGEN PLOTZ
The Effects of Cortisone Acetate on Intact and Adrenalectomized Rats During Pregnancy 384

SHELESNYAK, M. C.
Comparative Effectiveness of Antihistamines in Suppression of the Decidual Cell Reaction in the Pseudopregnant Rat 396

BACCHUS, HABEEB
Maintenance of Blood 17-Hydroxycorticosteroids Following Ascorbic Acid Treatment 402

HISAW, FREDERICK L. AND JOSEPH T. VELARDO
Influence of a Potentiator of Estrogen in the Inhibition of Progesterone in Development of Deciduomata 409

TABER, ELSIE AND KATHRYN W. SALLEY
The Effects of Sex Hormones on the Development of the Right Gonad in Female Fowl 415

LANGLEY, L. L., P. W. SCOKEL AND E. J. MOORE
Role of Hypoglycemia and Carbon Dioxide in the Reaction to Stress 425

MILLER, ZELMA
A Study of the Lymphocytolytic Action of an Adrenal Cortical Extract *in Vitro* 431

VANDERLAAN, W. P. AND RAYNOR CAPLAN
Observations on a Relationship Between Total Thyroid Iodine Content and the Iodide-Concentrating Mechanism of the Thyroid Gland of the Rat 437

CLEGG, M. T., J. M. BODA AND H. H. COLE
The Endometrial Cups and Allantochorionic Pouches in the Mare with Emphasis on the Source of Equine Gonadotrophin 448

JANES, RALPH G.
Effect of a Pituitary Gonadotrophin on the Ovaries of Hypothyroid Rats ... 464

NOTES AND COMMENTS

DICZFALUSY, EGON
Poly-Estradiol Phosphate (PEP); a Long-Acting Water Soluble Estrogen. 471

ESSELLIER, A. F., P. JEANNERET, E. KOPP AND L. MORANDI
Evidence Against Destruction of Eosinophils by Glucocorticoids as Shown by *In Vitro* Experiments 477

THE ENDOCRINE SOCIETY
The 1954 Annual Meeting 480

THE LAURENTIAN HORMONE CONFERENCE
The 1954 Annual Meeting 480

BOOK REVIEW 482

ISSUED MONTHLY FOR THE ENDOCRINE SOCIETY
BY CHARLES C THOMAS, PUBLISHER,
SPRINGFIELD, ILLINOIS

Annual Subscription $12.00 Single Copies $1.50

Questions and answers on

BLUTENE CHLORIDE

TRADE MARK

(TOLONIUM CHLORIDE, ABBOTT)

the new oral treatment for functional uterine bleeding

Q. What is Blutene?

Blutene is a new, non-hormonal antimenorrhagic—a specially purified form of toluidine blue O. It has proved effective in some cases where estrogens, thyroid extract, curettage, etc., have failed.

Q. How is Blutene administered?

Blutene is taken orally. One 100 mg. tablet b.i.d. (or t.i.d. in resistant cases) is usually effective.

Q. How long should Blutene be continued?

One treatment period not exceeding five days will end symptoms for some patients, though long-standing cases may require an additional course if excessive bleeding occurs in subsequent menses. Recurrences are relatively few.

Q. Can Blutene also be used as a preventive?

Yes. When the patient has had excessive functional uterine bleeding the previous month, the physician can prescribe Blutene five days before the next menses as a safeguard against recurrence.

Q. How does Blutene take effect?

Patients with menometrorrhagia often exhibit an increase of heparin-like substances in the blood. Blutene counteracts these excess heparinoid substances.

Q. Is Blutene well tolerated?

Usually.[1,2,3,4] Side effects of tenesmus or burning on urination—if noted at all—frequently respond to increased fluid intake; if persistent nausea is met, smaller doses may be better tolerated and still prove effective. Doses of 200 mg. daily are less likely to cause these difficulties.

Q. When should Blutene NOT be used?

IMPORTANT: Blutene *should not be used until gynecologic examination has ruled out carcinoma, blood dyscrasia, or other organic causes of bleeding.* The drug has no effect on these conditions, but valuable time would have been lost in starting required surgical or X-ray procedures. Abbott

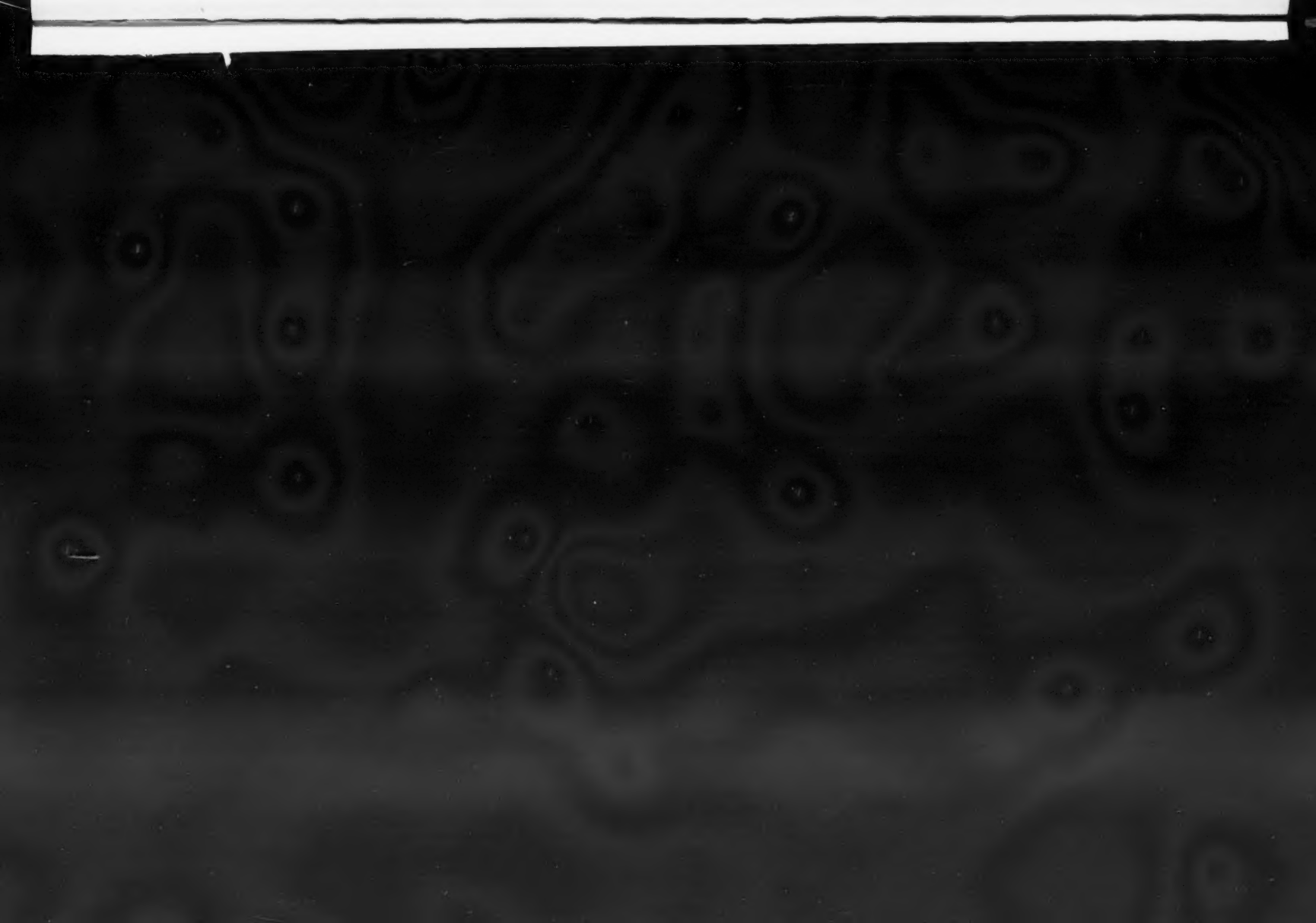
100 mg. tablets, bottles of 25 and 100.

Write for descriptive literature to

Abbott Laboratories, North Chicago, Ill.

1. Rumbolz, W. L., Moon, C. F., and Novelli, J. C., Use of Protamine Sulfate and Toluidine Blue for Abnormal Uterine Bleeding, Amer. J. Obst. & Gynec., 63:1029, May 1952.
2. Priddle, H., Discussion of Rumbolz et al., Amer. J. Obst., & Gynec., 63:1036, May, 1952.
3. Lathrop, C. A., and Carlisle, W. T., Oral Toluidine Blue in the Treatment of Hypermenorrhea, Amer. J. Obst. & Gynec., 64:1376, Dec., 1952.
4. Bickers, W., Toluidine Blue—An Evaluation in the Treatment of Uterine Bleeding, Amer. J. Obst. & Gynec., 66:1313, Dec., 1953.

454370



ENDOCRINOLOGY

VOLUME 54 APRIL, 1954 NUMBER 4

Copyright 1954 by the Endocrine Society

BIOASSAY OF ADRENAL STEROIDS IN BLOOD AND URINE BASED ON EOSINOPHIL RESPONSE: A STATISTICAL ANALYSIS

EUGENIA ROSEMBERG,[1] JEROME CORNFIELD,[2] ROBERT W. BATES[3] AND EVELYN ANDERSON[3]

National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland

SEVERAL methods, both chemical and biological, for the assay of adrenal steroids in body fluid have been described (Dorfman, 1949; Venning, 1950; Porter and Silber, 1950). There is, however, no satisfactory agreement in the quantitative measurement of the steroids by the different methods; this difference is especially striking between the chemical and biological methods. New and better methods for measuring the physiological activity of the steroid hormones in blood and urine are consequently exceedingly important. The present paper is devoted to discussing an assay method based upon the eosinophil response of adrenalectomized mice, indicating the conditions necessary for satisfactory performance, studying the factors influencing the error of the method, and comparing results with those obtained with other methods.

LABORATORY METHODS

Male mice (C-57 Brown/edJN strain), weighing 20–22 gms., were adrenalectomized and a pellet of DCA (15 mgms.) was implanted subcutaneously. For the first two days after operation the animals were kept in an incubator at 28° C. and given 5% glucose in normal saline to drink. After that, they were kept in an air conditioned room at 25° C. and given tap water and *Purina Fox Chow*.

To test for completeness of adrenalectomy the response of the eosinophil cells to 0.5

Received for publication August 4, 1953.

[1] Post Doctorate Fellow, Public Health Service. Present address: Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts.

[2] Office of Biometry, National Institutes of Health.

[3] Section on Endocrinology, National Institute of Arthritis and Metabolic Diseases.

U.S.P. Units of ACTH intraperitoneally was used. The procedure was the same as for the assay described below. If any fall in eosinophil count occurred in the 4-hour period after injection the animals were discarded. The animals could be reused every 4 days. They were checked with ACTH every 10 days to rule out regrowth of adrenal tissue. The technique of collecting blood, staining and counting of the eosinophil cells is that described previously (Rosemberg and Lewis, 1950) except that in counting, as many squares were counted as was necessary to obtain at least 50 cells.

Cortisone acetate was used as the reference standard. A stock solution (100 μg./ml.) was made by dissolving cortisone acetate in a mixture of equal parts of warm ethyl alcohol and propylene glycol. The test solutions were made by diluting the stock solution with a mixture of equal parts of propylene glycol and saline and were administered intraperitoneally.

Urine was kept in the refrigerator without preservative during the 24-hour collection period. The urine was then adjusted to pH 7, subdivided into 10-ml. aliquots and frozen. By thus inhibiting growth of bacteria no toxic effects were apparent when 2 ml. of crude urine were injected intraperitoneally into the test mice.

Cortical steroids were extracted from heparinized plasma with a mixture of 2 parts of ethyl acetate and one part of ethyl ether. The fat was removed from the residue obtained on evaporation of this extract by partitioning between hexane and 70% ethanol. The residue on evaporation of the 70% ethanol was dissolved in a small volume (0.4–5 ml.) of 50% propylene glycol for injection.

ASSAY METHOD AND STATISTICAL ANALYSIS

Conduct of Assay:

(a)	8:30 A.M.	Epinephrine 5 μg. injected intraperitoneally.
(b)	11:30	Blood taken for first eosinophil count.
(c)	11:31	Intraperitoneal injection of the material to be tested.
(d)	3:30	Blood taken for second eosinophil count.

For urine samples a constant volume of 2 ml. was injected, and the test solution of the standard was diluted with saline to give the same volume. For plasma extracts either 0.2 or 0.4 ml. was injected and the standard administered in the same volume.

Establishing the dose-response curve

Each of 40 mice, treated as described above, was tested at each of five dose levels of cortisone, the interval between tests on each mouse being one week. For each animal the test consisted of steps (a–d) above and (e) computation for each animal of the per cent change in eosinophil count, i.e.

$$\frac{\text{second count} \times 100}{\text{first count}} - 100 = \% \text{ change.}$$

Each dose level was a three-fold dilution of the next higher level, the five doses used being 20 μg., 6.67 μg., 2.22 μg., 0.74 μg., 0.247 μg. To balance out possible effects of previous injection, or day of experiment, the animals were arranged in eight Latin squares of five animals each. Thus, at each dose level one-fifth of the animals had received no previous inoculation of cortisone, one-fifth had received one previous inoculation, etc.

The results of this experiment are shown in Figure 1. Each observed point is an estimated median per cent change in eosinophil count for the 40 mice tested at each dose

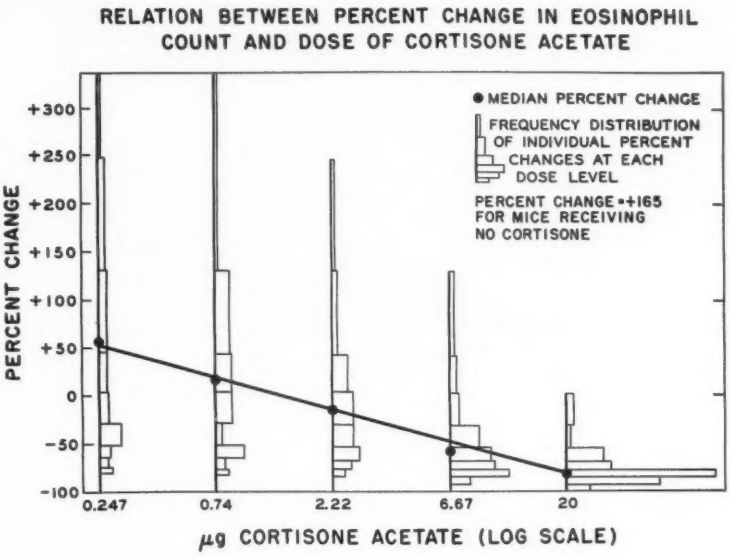


FIG. 1

level. The relation is clearly linear against log dose, at least below 10 μg., and provides the basis for an essay.

We have also shown in Figure 1 the frequency distribution of individual per cent changes in eosinophil count at each dose level. It will be noted that for all dose levels below 20 μg. some test animals showed increases in count, and that below 2 μg. more than half showed such increases. We have consequently not found it possible to follow the practice of previous studies relating changes in eosinophil counts to dosage of adrenal steroids, in which only decreases in counts are considered and increases are included as zero decreases. Each observed point on the figure is an estimate of the median per cent change in count, with increases and decreases accorded equal weight.

How can increases in eosinophil count in response to a dose of cortisone provide information on the amount of cortisone present when the characteristic effect of cortisone is to decrease the count? In Figure 2 we show the change from 11:30 to 3:30, the standard test period, in eosinophil count for 28 test animals given no cortisone but pretreated with epinephrine. All but 4 show increases in count, the median change being +158%, or well above that found after 0.247 μg. cortisone in Figure 1. In Table 1 we show the response of test animals to handling, loss of blood, placebo injections, and epinephrine in the absence of cortisone. In every case the change is upward. The justification therefore for considering increases as a measure of response is that in the absence of hormone and under the conditions of the present experiment (particularly pretreatment with epinephrine) the characteristic eosinophil response of adrenalectomized animals is a substantial increase in count. Cortisone decreases the magnitude of this increase, but only at the higher dose levels does it decrease it sufficiently to convert increases to decreases.

In Figure 3 we show what happens to the dose-response curve of Figure 1 when all

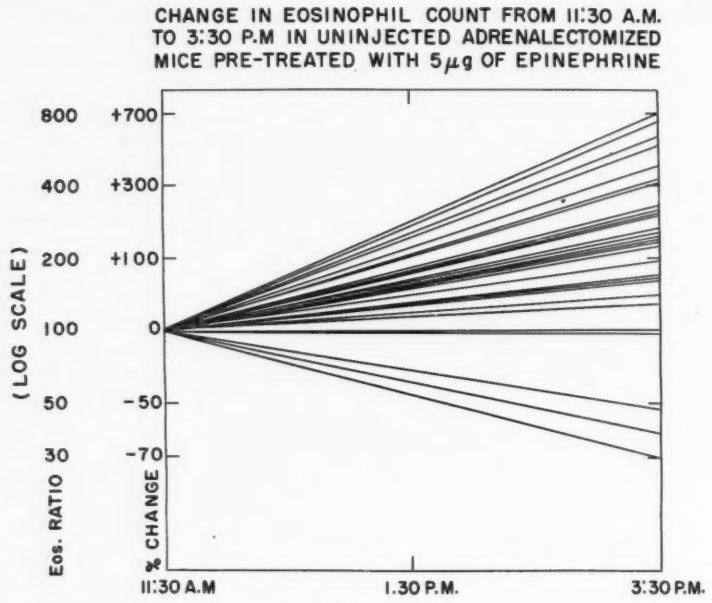


FIG. 2

increases are treated as zero decreases. The linear relationship is of course seriously impaired and the range of sensitivity of the assay decreased by approximately tenfold.

Statistical characteristics of response

The distributions of animals by per cent change in count at each dose level shown in Figure 1 are skewed. An arithmetic average of the individual per cent changes would consequently be markedly affected by the changes found for one or two animals. Thus, at 0.247 μg. the largest increase observed was +387%. Even with 40 test animals the effect of this one animal on the arithmetic average would be large, raising it by almost 10 percentage points. The arithmetic average would consequently yield unstable

TABLE 1. EOSINOPHIL RESPONSE OF ADRENALECTOMIZED MICE WITH AND WITHOUT PRETREATMENT WITH EPINEPHRINE

Injection	Pretreatment	Total number of animals	Number of animals showing fall in eosinophil count	Median per cent change	68% confidence limits
None	None	28	5	+ 71	(82–50)
0.1 ml. 50% propylene glycol and saline	None	27	7	+ 65	(77–46)
None	5 μg. of epinephrine	28	4	+158	(187–112)
0.1 ml. 50% propylene glycol and saline	5 μg. of epinephrine	28	2	+173	(206–148)

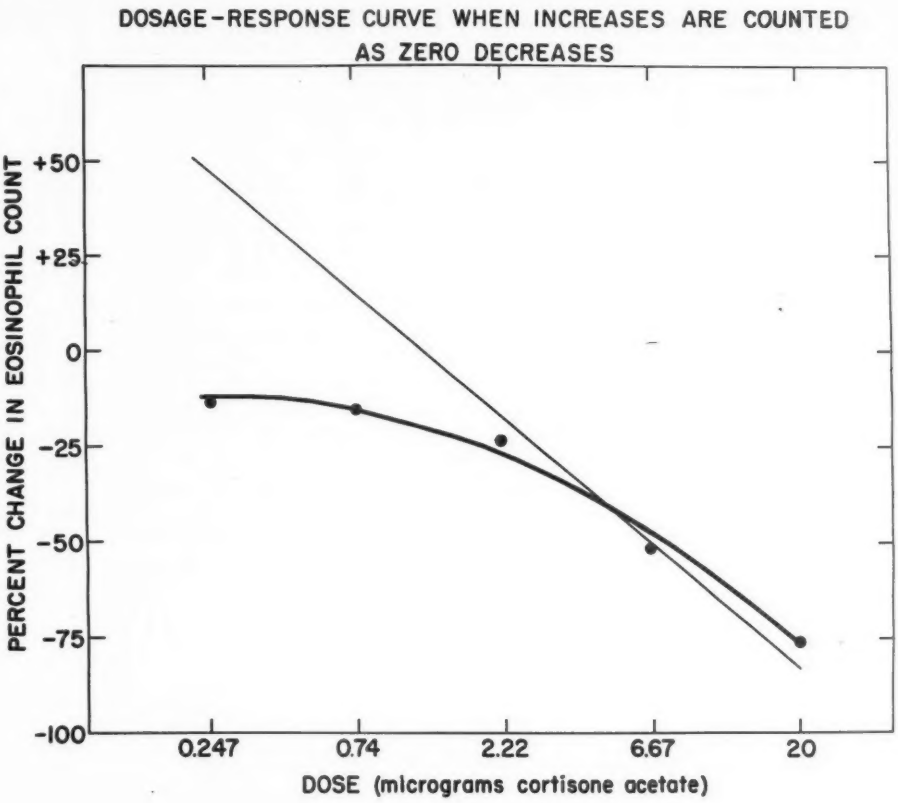


FIG. 3

average per cent changes and an inaccurate assay. The median per cent change, defined as that per cent change which is exceeded by half the animals and which in turn exceeds the change shown by the other half, will clearly be much less affected by very large increases. The endpoint chosen has therefore been the median per cent change. Our actual computing procedure, however, (vide infra) has involved estimating the median by

(a) Computing the ratio of the 3:30 to the 11:30 count for each animal
(b) Taking the geometric mean of these ratios
(c) Expressing this mean ratio as a percentage change by multiplying it by 100 and subtracting 100 from the product.

We refer in what follows to the ratio $\times 100$ (or what amounts to the same thing, the percentage change $+100$) as the eosinophil ratio. The relationship between the mean eosinophil ratio and the mean percentage change is indicated by the double ordinate scale in Figure 2. The justification for this calculating procedure is that the eosinophil ratios are approximately log normally distributed (as are many other biological variables). For data so distributed the calculating procedure used provides as accurate an estimate of the median per cent change as can be made.

TABLE 2. GEOMETRIC MEAN OF EOSINOPHIL RATIOS AND STANDARD DEVIATION OF LOGARITHM OF EOSINOPHIL RATIOS FOR EACH MOUSE AT DIFFERENT DOSE LEVELS

Cortisone	Geometric mean	Logarithmic S.D. $=\sigma$	Antilog (log S.D.) 10^{σ}
μg.			
20	18	0.344	2.2
6.67	47	0.382	2.4
2.22	87	0.312	2.1
0.74	117	0.330	2.1
0.247	139	0.256	1.8
all doses combined	—	0.327	2.1

One other characteristic of the response should be noted. From Figure 1 it is clear that the distributions show much less variation at the high dose levels than at the low ones. We show in Table 2 the standard deviation of the logarithms of the eosinophil ratios at each dose level for the data shown in Figure 1. It will be noted that the logarithmic standard deviations are approximately equal at all dose levels. We have taken advantage of this by pooling the variance at each dose level thus obtaining a single estimate of the logarithmic standard deviation for each experiment.

For normally distributed data approximately 68% of the cases are included in the interval (arithmetic mean−standard deviation) to (arithmetic mean+standard deviation). For data which are log normally distributed approximately 68% of the cases are included in the interval [geometric mean ÷antilog (log st. dev.)] to [geometric mean ×antilog (log st. dev.)] (e.g. $18 \div 2.2 = 8$ to $18 \times 2.2. = 40$ for the 20 μg. level). Throughout this paper 68% confidence limits on bioassay results are used.

Variations in standard

An important question in any assay is whether the response to the standard changes from time to time. If it does not, a single standard curve, such as that shown in Figure 1, can be determined once and for all. The eosinophil count is affected by factors other than cortisone, however, and to the extent that these other factors vary, the standard curve might also be expected to vary. When response to the standard varies, referring responses for an unknown to a single unchanging standard curve introduces errors of assay that may be large, and that cannot in any event be evaluated from the internal evidence of the assay. To check on the possibility of a varying response to the standard we have made simultaneous determinations on standard and unknown in most of the assays we have made. The responses to the standard in these assays are assembled in chronological sequence in Table 3 in which we show the eosinophil ratio at each dose level and the 68% confidence limits on this ratio. It is apparent that response to the standard varies significantly from assay to assay. The table also shows, however, that the response to 10 μg. is essentially unchanged, and that the curve appears to swivel about a fixed response at that point. We have been unable to identify any of the factors responsible for these changes in response, and consequently conclude that simultaneous determinations on the standard at one dose level below 10 μg. are a necessary precaution in this assay.

Design and error of an assay

It is customary to describe the inherent precision of an assay by λ, defined as the ratio of the standard deviation of responses to the slope of the dose response curve. This is not an entirely satisfactory procedure for the present assay for several reasons, but for comparative purposes we compute some approximations to λ first.

TABLE 3. EOSINOPHIL RESPONSE TO CORTISONE ACETATE IN SUCCESSIVE ASSAYS

10 μg.			3.16 μg.			1 μg.		
No. of animals	Eosinophil ratio*	68% confidence limits	No. of animals	Eosinophil ratio*	68% confidence limits	No. of animals	Eosinophil ratio*	68% confidence limits
7	16	(20–13)	7	24	(30–20)	7	48	(60–39)
7	23	(29–19)	7	51	(63–41)	7	84	(104–67)
7	15	(19–11)	7	43	(56–33)	7	73	(96–56)
7	17	(24–12)	7	46	(63–33)	7	92	(128–66)
10	14	(19–11)				10	103	(134–79)
10	18	(24–14)				10	132	(177–98)
10	29	(35–24)				12	127	(153–105)
10	18	(24–16)				10	157	(193–129)
10	15	(18–13)				10	122	(148–101)
10	12	(15–10)				5	313	(426–230)
15	22	(25–19)				15	140	(162–121)
10	16	(21–12)				12	216	(274–170)

* Geometric mean of eosinophil ratio.

If we denote by σ the logarithmic standard deviation of Table 2, by y the geometric mean eosinophil ratio and by n the number of observations on which the mean ratio is based, then the standard error of y as given by Finney (1941) is

$$\text{S.E.}(y) = y\sqrt{10^{\sigma^2/n}(10^{\sigma^2/n} - 1)} . \tag{1}$$

If we denote the slope of the dose response curve (change in eosinophil ratio/tenfold dilution) by b, we then have for

$$\lambda = \frac{y\sqrt{10^{\sigma^2}(10^{\sigma^2} - 1)}}{b} . \tag{2}$$

The values of λ for the assays shown in Figure 1 and Table 3 are shown in Table 4. The values shown indicate that for unknowns containing the equivalent of 10 μg., the assay

TABLE 4. ESTIMATES OF λ AT DIFFERENT LEVELS OF CORTISONE IN DIFFERENT EXPERIMENTS

No. mice	$\sqrt{10^{\sigma^2}(10^{\sigma^2}-1)}$	b	Equivalent of cortisone in dose administered			
			10 μg.	5 μg.	2 μg.	1 μg.
			λ	λ	λ	λ
200*	0.60	65.5	0.36	0.54	0.77	0.95
21	0.41	36.7	0.16	0.28	0.45	0.57
21	0.43	60.4	0.16	0.29	0.46	0.59
21	0.55	60.8	0.13	0.29	0.51	0.68
21	0.74	64.4	0.20	0.42	0.70	0.93
20	0.70	88.5	0.11	0.33	0.60	0.82
20	0.82	113.7	0.13	0.38	0.70	0.95
22	0.49	98.0	0.14	0.29	0.49	0.64
20	0.50	138.6	0.07	0.22	0.42	0.57
20	0.45	106.7	0.06	0.20	0.38	0.51
15	0.53	300.3	0.02	0.18	0.39	0.56
30	0.41	118.1	0.08	0.20	0.36	0.49
22	0.68	200.0	0.05	0.26	0.53	0.74
Ave.			0.13	0.30	0.52	0.69
±S.E.			±0.03	±0.03	±0.03	±0.04

* 40 mice each used 5 times.

has a satisfactory precision, while for unknowns with less than 1 μg., the precision is low, and many more animals would be required to obtain an equally accurate assay. While the assay can detect the presence of amounts at least as low as 0.247 μg., as indicated by Figure 1, the practical limit of the assay can be taken as 1 μg. because of the high value for λ.

What we really require is an indication of the number of animals needed to attain a predetermined degree of precision. Before we can do this, we must determine the way in which an assay of an unknown is to be designed. If we could safely assume that (a) the standard remained unchanged (b) the dose response curves for standard and unknown remained parallel and linear, one dose level for the unknown would be sufficient. If we can assume none of these, then three dose levels for both standard and unknown would be required (Perry, 1950). In the usual assay in which variation is the same at all dose levels, the accuracy of the estimate obtained for the unknown is not seriously affected by whether a given number of animals is distributed over 6 dose levels, permitting the required checks, or over a smaller number, thus waiving some of them. In the present assay, any check on linearity or parallelism requires animals at low dose levels, where variation is higher, thus impairing the accuracy of the estimate. We discuss parallelism more fully later and merely note here that we are unwilling to check routinely on departures from parallelism and linearity at the expense of the precision of the estimate that the assay is designed to yield.

We are compelled, however, by the results shown in Table 3, to make simultaneous determinations on the standard. This means we must have one dose level for the unknown and one for the standard. The design that seems suited to the conditions under which our experiments have been performed therefore appears to require a group of animals tested with the standard at 1 μg. and another group tested at one dose level of the unknown. If we take response to 10 μg. of the standard as 18 (vide Table 3), denote the eosinophil ratio for the standard at 1 μg. by $\overline{S}$, the eosinophil ratio for the unknown by $\overline{U}$, and log equivalent μg. of cortisone acetate by M, then

$$M = \frac{\overline{S} - \overline{U}}{\overline{S} - 18} \tag{3}$$

while

$$\text{equivalent } \mu\text{g. of cortisone} = 10^{(\overline{S}-\overline{U})/(\overline{S}-18)} = 10^{M}.$$

If we use n animals for the standard and n for the unknown[4] then

[4] The variance can be made smaller by taking

$$\frac{2n}{1 + \left[\frac{\overline{U}(\overline{S} - 18)}{\overline{S}(\overline{U} - 18)}\right]^2}$$

animals for the standard and

$$\frac{2n}{1 + \left[\frac{\overline{S}(\overline{U} - 18)}{\overline{U}(\overline{S} - 18)}\right]^2}$$

animals for the unknown, which reduces to equality when the unknown contains 1 μg., in which case $M = 0$. When a good guess of M can be made in advance of the assay and M is well above 0, this allocation will increase precision.

(4) the standard error of M, $\text{S.E.}(M) \cong \sqrt{\frac{10^{\sigma^2/n}(10^{\sigma^2/n}-1)}{\overline{S}-18}}\sqrt{(1-M)^2\,\overline{S^2}+U^2}$.

Approximate 68% confidence limits to M (log equivalents cortisone) are then given by $M-\text{S.E.}(M)$, and $M+\text{S.E.}(M)$. The antilogs of these quantities give approximate 68% confidence limits on the amount of cortisone equivalents.

These limits may be written as:

(5) $10^{M} \div 10^{\text{S.E.}(M)}$ and $10^{M} \times 10^{\text{S.E.}(M)}$.

In Table 5 we show the confidence limits obtained from this formula for varying numbers of animals and for varying cortisone dosage: When the dose is 10 μg., the percentage errors are low, even with small numbers of animals. Five animals for the standard and 5 for the unknown results in only a 10% error. When the dose is only one tenth or 1 μg. of cortisone it is not possible to obtain such small percentage errors even with 25 animals.

TABLE 5. 68% CONFIDENCE LIMITS FOR ESTIMATED CORTISONE EQUIVALENTS FOR DIFFERING NUMBERS OF ANIMALS AND DIFFERING AMOUNTS OF CORTISONE IN UNKNOWN

Number* of animals	Cortisone equivalents in dose of unknown					
	10 μg.		5 μg.		1 μg.	
	Confidence limits					
	Upper†	Lower‡	Upper	Lower	Upper	Lower
	μg.	μg.	μg.	μg.	μg.	μg.
5	11.0	9.1	6.8	3.7	2.3	0.4
10	10.7	9.4	6.2	4.0	1.8	0.6
15	10.5	9.5	5.9	4.2	1.6	0.6
20	10.5	9.6	5.8	4.3	1.5	0.7
25	10.4	9.6	5.7	4.4	1.4	0.7

* n of equation (4).
† Equivalent μg. $\times 10^{\text{S.E.}(M)}$
‡ Equivalent μg. $\div 10^{\text{S.E.}(M)}$

To what extent do standard errors calculated from the internal evidence of a single assay really provide an indication of how much results would vary if a large number of independent assays were performed on the same unknown? The first three assays of Table 12 were independent assays of the same urine. It will be noted that they do in fact agree within the limits of error calculated for each of the three assays.

Miscellaneous considerations

Reuse of animals.—The dose response curve of Figure 1 is essentially the average of 5 different curves obtained at weekly intervals by using the same 40 animals over and over again. In the first week none had received any previous injections of cortisone, in the second week all had been previously injected once, etc. We show in Table 6 the 5 separate dose response curves obtained. It will be seen that number of previous injections made no difference in the curve obtained. Sensitivity is as high after the fifth week as it was at the beginning.

Effect of epinephrine.—The effect of pretreatment with epinephrine was studied by repeating the experiment summarized in Figure 1, except that pretreatment with epinephrine was withheld. The results are summarized in Table 7. Comparison of these

TABLE 6. EOSINOPHIL RATIO AT DIFFERENT DOSE LEVELS FOR DIFFERENT NUMBERS OF PREVIOUS INJECTIONS WITH CORTISONE

Number of previous injections with cortisone	Cortisone acetate injected				
	20 μg.	6.67 μg.	2.22 μg.	0.74 μg.	0.247 μg.
	Eosinophil ratio*				
0	17	44	86	119	122
1	17	44	65	164	138
2	31	53	85	61	185
3	21	51	91	125	59
4	11	44	128	149	179

* Geometric mean for eight animals at each dose level.

results with those shown in Figure 1 and Tables 3 and 6 shows that the slope is considerably reduced when epinephrine is omitted, although the variation is essentially unaltered.

Similarly, reference to Table 1 indicates that, in the absence of cortisone, animals pretreated with epinephrine show much larger eosinophil ratios for the 4 hour period than those not so treated. The steeper slope obtained with cortisone in epinephrine treated mice is thus due largely to the fact that epinephrine produces larger eosinophil ratios in the absence of cortisone. This effect of pretreatment with epinephrine on the eosinophil ratio is due largely to a lowering of the 11:30 A.M. eosinophil count. With the variation essentially unchanged by the epinephrine, the dose response curve with the steeper slope gives a lower λ value and hence a more accurate assay.

Error in eosinophil count.—A study of the errors in our counts suggests an error well in excess of the Poisson variation reported by other investigators. We have made no systematic attempt to identify the sources of this excess variation because calculation indicates that the variation in the eosinophil ratios would be only slightly reduced if the counting error could be reduced to its theoretical minimum. The major source of variation appears to be among animals and not in the error of counting.

Hematocrit correction.—The injection of 2 ml. of urine into a test animal resulted in about a 15% decrease in hematocrit after 4 hours, with only moderate variation from animal to animal. This means that the blood has been diluted and that animals injected with cortisone-free urine would show a 15% lower eosinophil count than would uninjected animals. The injection of 2 ml. of normal saline with or without cortisone, however, results in no decrease in hematocrit. To correct for this, all of the 3:30 P.M. counts in the animals injected with urine were increased by 15%. Until further information is available on how this hematocrit effect varies among individual

TABLE 7. EOSINOPHIL RATIO AT DIFFERENT DOSAGES OF CORTISONE WITHOUT PRETREATMENT WITH EPINEPHRINE

Cortisone, μg.	Eosinophil ratio	68% confidence limits	
		Upper	Lower
20	14.9	17.6	12.6
6.67	26.9	30.5	23.9
2.22	46.2	53.4	40.0
0.74	57.9	68.4	49.0
0.247	94.0	108.0	81.7

urines, it might be safer to compute separate hematocrit corrections for each urine assayed.

Parallelism of two curves.—An important question in any assay is the parallelism of the dose response curves for standard and unknown. The first four urine assays were designed to answer this question. Three dose levels were used for the unknown and three for the standard. There were seven animals at each dose level or 42 in each assay. Three assays were done on aliquots of a single 24 hour collection of urine and a fourth assay on another 24 hour collection of urine from the same individual. Both the standard and unknown were diluted with saline when necessary to bring the total volume injected up to 2 ml. The slopes obtained are shown in Table 8. The average for the standard, 53, differs but little from that for the unknown, 62. The results of the urine assays are given in the next section.

A test for parallelism of the curves for blood plasma from the adrenal vein of a dog and for the standard is indicated in Figure 4.[5] Three dose levels were used, equivalent to 0.5, 1.0, and 5.0 ml. of plasma. The highest dose level gave a median decrease of 90%, which from the figure is outside the interval of linearity for the standard. The two lower

TABLE 8. SLOPES (CHANGE IN EOSINOPHIL RATIO/10 FOLD DILUTION) FOR STANDARD AND URINE IN FOUR EXPERIMENTS

Exp. no.	Standard	Urine
1	28	67
2	58	55
3	58	71
4	67	54
Ave.	53	62

dose levels afford no indication of departure from parallelism. These checks are not routinely performed because they require the use of low dose levels and, as indicated in the section on errors, low dose levels require large numbers of animals for satisfactory accuracy. The levels of cortisone equivalents obtained in this assay are reported in the section *Results with Bioassay.*

Calculating procedure

We present in Table 9 an illustrative computation of cortisone equivalents for a single assay. Columns (1) and (2) present the eosinophil counts made before and after the injection of 2 ml. of urine in each of 23 mice. Columns (5) and (6) present the same information for 12 mice injected with 2 ml. of saline containing 1 μg. of cortisone acetate. In columns (3) and (7) the individual eosinophil ratios are shown, while in columns (4)

[5] The blood was collected in the manner described in connection with the series of assays reported in Protocol II (vide infra). In order to insure a sufficiently high level of endogenous adrenal steroids, 8 I.U. ACTH were injected intravenously at the start of the collection, and over the next four hours ACTH was added every hour to the blood transfusion bottle; a total of 60 I.U. ACTH was given. A transfusion of 400 cc. of whole blood (normal canine serum and the animal's own blood cells) was given over the four hour period during which time 347 ml. of blood was withdrawn from the adrenal vein. The plasma was extracted as described in the section on *Laboratory Methods.*

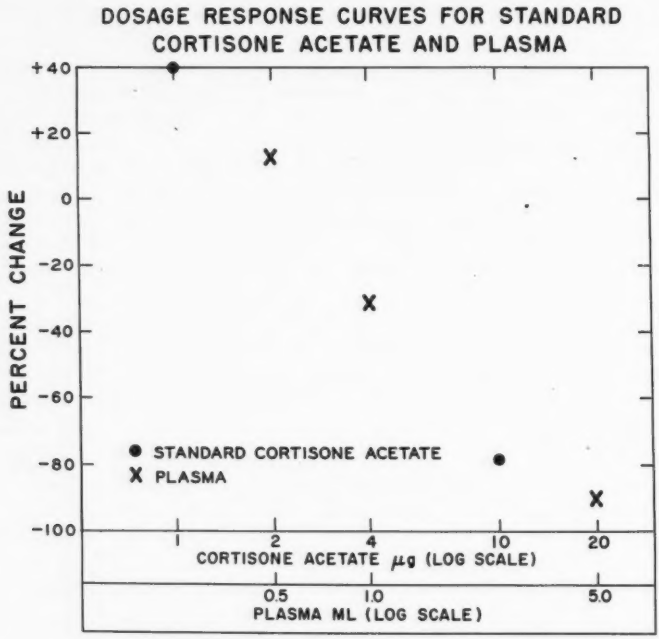


FIG. 4

and (8) we show the logs of these ratios. The block "Computation procedures" shows the systematic method routinely used to compute cortisone equivalents. On line *A* we enter in the column headed "value" the number of animals used for the unknown, 23, and on line *B* the number used for the standard, 12. On line *C* we enter the sum of the logs in column (4), on line *D* the sum of column (8). On line *E* we enter the results of dividing the entry on line *C* by that on line *A* (48.585 ÷23 = 2.1124). Line *F* is similarly calculated. The calculation on line *G* (hematocrit correction) is the equivalent of raising each 3:30 count for the unknown by 15% and is used for urine, but not for plasma or standard. The antilogs of the entries on lines *F* and *G* give the geometric means which are entered in lines *H* and *I*. The calculation on line *J* gives the estimate of log cortisone equivalents according to equation (3). The antilog of the entry in line *J*, entered on line *K*, gives the cortisone equivalents.

For a simpler, but not so precise, calculating procedure, one can substitute medians for geometric means. The median ratio for the 23 mice injected with the unknown is 119, which when increased by 15% for hematocrit becomes 136.9 as compared with a geometric mean of 148.9. The median for the standard is 128.5 compared with a geometric mean of 126.8. When one wishes to avoid taking logs, and is willing to accept a less precise estimate, these medians may be substituted for the geometric means in lines *H* and *I*. The method of computing cortisone equivalents is unaffected.

The computation of 68% confidence limits on the estimated equivalent cortisone according to formula (5) is shown in Table 10, which is self-explanatory. Lines *c* and *d* give the required confidence limits. When one routinely computes confidence limits on

TABLE 9. ILLUSTRATIVE COMPUTATION OF CORTISONE EQUIVALENTS

Unknown				Standard 1 μg.			
Eosinophil count per mm.³		Esinophil ratio	Log of ratio	Eosinophil count per mm.³		Eosinophil ratio	Log of ratio
11:30	3:30	(2)/(1) ×100	log (3)	11:30	3:30	(2)/(1) ×100	log (7)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
268	313	117	2.068	63	50	79	1.898
153	128	84	1.924	25	35	140	2.146
97	241	248	2.394	94	75	80	1.903
144	103	76	1.880	84	119	142	2.152
106	100	94	1.973	181	203	112	2.049
38	113	297	2.473	144	235	163	2.212
50	131	262	2.418	327	343	112	2.049
178	197	111	2.045	88	308	350	2.544
203	275	135	2.130	159	186	117	2.068
206	283	137	2.137	213	126	59	1.771
78	371	476	2.678	156	236	151	2.179
327	189	58	1.763	209	386	185	2.267
97	436	449	2.652				
567	106	19	1.279				
153	300	196	2.292				
186	207	111	2.045				
184	242	132	2.121				
91	66	73	1.863				
223	364	163	2.212				
347	227	65	1.813				
172	181	105	2.020				
116	247	213	2.328				
100	119	119	2.076				

COMPUTATION PROCEDURES

Entry		Designation	Calculation	Value
no. of mice,	*U**	*A*	—	23
no. of mice,	*S*†	*B*	—	12
sum of logs,	*U*	*C*	Σ(4)	48.585
sum of logs,	*S*	*D*	Σ(8)	25.238
logarithm ave.,	*U*	*E*	$C \div A$	2.1124
logarithm ave.,	*S*	*F*	$D \div B$	2.1032
hematocrit correction,	*U*	*G*	$E + 0.0605$	2.1729
geometric mean,	*U*	*H*	Antilog *G*	148.9
geometric mean,	*S*	*I*	Antilog *F*	126.8
log cortisone equivalents		*J*	$(I - H) \div (I - 18)$	−0.2031
cortisone equivalents		*K*	Antilog *J*	0.626

* Unknown.
† Standard.

all estimates of cortisone equivalents, it is more convenient to combine both tables into one.

EXPERIENCE WITH BIOASSAY METHODS

The previous section provides a statistical evaluation of the assay. In order to arrive at a biological evaluation of the method, a series of experiments were set up in which adrenal steroids in blood were assayed both

TABLE 10. ILLUSTRATIVE COMPUTATION OF 68% CONFIDENCE LIMITS ON EQUIVALENT CORTISONE

Entry		Designation	Computation	Value
Sum of squares,	*U*	L	$\Sigma(4)^2$	104.7320
Sum of squares,	*S*	*M*	$\Sigma(8)^2$	53.5221
		N	$(L+M)-(C\times E+D\times F)$	2.5426
logarithmic variance		*O*	$N\div(A+B-2)$	0.07705
logarithmic (S.E.)^2,	*U*	*P*	$O\div A$	0.003350
logarithmic (S.E.)^2,	*S*	*Q*	$O\div B$	0.006420
	U	*R*	Antilog *P*	1.0078
	S	*S*	Antilog *Q*	1.0149
	U	*T*	$R(R-1)$	0.0079
	S	*U*	$S(S-1)$	0.0151
	U	*V*	$T\times H^2$	175.0
	S	*W*	$U\times I^2$	243.0
		X	$W(1-J)^2$	352.0
		Y	$\sqrt{V+X}$	23.0
S.E. [log. cort. equiv.]		*Z*	$Y\div(I-18)$	0.2114
		a	$J+Z$	0.0083
		b	$J-Z$	−0.4145
68% confidence limits		*c*	Antilog *a*	1.02
		d	Antilog *b*	0.38

before and after ACTH and after hypophysectomy. Assays were also made on blood and urine of normal men and women and on the blood and urine of a patient with Cushing's disease. In 3 instances a chemical assay was made on the same extract of blood for comparison with the bioassay. The purpose of these experiments was to test the assay procedure in a variety of situations and not to initiate new studies or obtain normal values.

Recovery of cortisone added to blood.—Sixty ml. of blood were drawn from a leg vein of a normal fasted dog. To 30 ml. of this blood, 40 μg. cortisone acetate (0.4 ml. of the standard stock solution) were added. The 2 aliquots were extracted by the method described above. The sample with the cortisone acetate added was assayed using 4 mice; the other sample using 2 mice.[6] The findings were as follows:

	Total cortisone equivalents, μg.
30 ml. blood + 40 μg. cortisone acetate	36 (40–32)*
30 ml. blood	2 (5–0.6)
Difference	34

* 68% confidence limits.

[6] In general the number of animals that could be used in the assay was limited by the amount of cortisone available in the plasma. For instance, when only 10 μg. of cortisone equivalents are available for assay, one could give each of 2 test animals 5 μg. or each of 10, 1 μg. Because the variation in response is greater at 1 μg., 2 test animals, each at 5 μg., actually yield a more accurate estimate than 10 at 1 μg.

Thus there is no evidence that any loss in extraction occurred but the error of the assay leaves open the possibility that as much as 25% may have been lost.

Levels of cortisone equivalents in peripheral venous blood, and in adrenal vein blood of dogs before and after ACTH.—A series of assays was done on the blood of a normal female dog studied at different times over a period of weeks. Blood was drawn from a leg vein, the lower part of the inferior vena cava and from the left lumbo-adrenal vein, before and after ACTH. The animal was fasted 20 hours before taking blood. The data are given in Protocol I.

PROTOCOL I. DOG #66, FEMALE, WEIGHT: 15 KG.

Date	Time		Cortisone equivalents
		Peripheral venous blood	*μg./100 ml. plasma*
10/21/52	10:00 A.M.	30 ml. blood from leg vein, whole blood extracted, assayed in 2 mice	15 (26–4)*
10/27/52	10:15 P.M.	ACTH, 150 I.U. in oil, intramuscular	
10/28/52	10:30 A.M.	30 ml. blood from leg vein, plasma extracted, assayed in 4 mice	44 (84–16)
	4:15 P.M.	ACTH, 150 I.U. in oil, intramuscular	
10/29/52	10:00 A.M.	90 ml. blood from lower inferior vena cava, plasma extracted, assayed in 4 mice	44 (57–31)
1/12/53	10:00 A.M.	120 ml. blood from leg vein, plasma extracted in 4 mice	9 (15–4)
		Adrenal vein blood	
12/ 9/52	10:30 A.M.	Anaesthetized with nembutal; polyethylene tube inserted into left lumbo-adrenal vein; 120 ml. saline+heparin infused into leg vein during operation	
	11:40 A.M.	Clamp on lumbo-adrenal vein near vena cava and *24 ml. blood* collected through tube in 15 minutes. 40 ml. saline+heparin infused into leg vein during this time; whole blood extracted, assayed in 2 mice	516 (688–328) (4.1 μg./minute)
	12:00 noon	ACTH 25 I.U. in 25 ml. saline infused for 8 minutes and blood collected from adrenal	
	12:21 P.M.	Blood collection stopped; total of 29 ml. in 21 minutes; whole blood extracted, assayed in 7 mice	494 (720–296) 3.5 μg./minute

* 68% confidence limits.

Another series of assays was done on adrenal vein blood of a normal female dog before and after ACTH administration. The animal was anaesthetized with nembutal and the left lumbo-adrenal vein cannulated with polyethylene tubing. On ligating the vein at its junction with the inferior vena cava, the direction of the blood flow was reversed so that it flowed through the tube into a 50 ml. centrifuge tube. Collections of blood over 20-minute intervals were made continuously for 5 hours. The blood was centrifuged as soon as possible, and after drawing off the plasma, the blood

PROTOCOL II. DOG #92, FEMALE, WEIGHT: 7.8 KG.

Time	Blood flow left adrenal vein ml./minute	No. mice given plasma extract	Cortisone equivalents	
			Rate μg./minute	Concentration μg./100 ml. plasma
10:50–11:30	2	6	0.93	93 (96–59)*
11:30–11:50	Lost			
11:50–12:50	2.5	4	0.95	78 (83–68)
12:50– 1:50	2.23	4	2.1	165 (211–120)
ACTH started. 5 mg. in 100 cc. blood transfused during the next hour.				
1:50– 2:10	1.8	5	1.79	156 (193–112)
2:10– 2:30	1.65	3	1.41	128 (155–104)
2:30– 2:50	1.45	3	1.09	109 (140–80)
5 mg. ACTH in 130 ml. blood transfused during the next hour.				
2:50– 3:10	1.7	3	0.76	66 (95–42)
3:10– 3:30	1.2	2	0.60	85 (114–52)
3:30– 3:50	0.7	2	0.65	138 (189–87)
Animal died quite suddenly at 3:52 P.M.				

* 68% confidence limits.

cells were added to normal canine serum and returned to the animal by a continuous transfusion. Heparin was used to prevent clotting. The transfused blood was injected at the same rate as the blood flow from the adrenal vein. The data of this experiment are given in Protocol II.

In the previous section under the heading *Parallelism of two curves* the bioassay on adrenal vein boood of a normal dog after ACTH administration was described. A chemical assay on the same extract of the blood samp'e was performed by Dr. Don H. Nelson by the method of Nelson and Samuels (1952). The results were as follows:

No. mice given plasma extract	Bioassay	Chemical assay
	μg./100 ml. plasma	μg./100 ml. plasma
15	383 (508–268)*	720

* 68% confidence limits.

Another experiment was carried out in which the left adrenal vein of a hypophysectomized dog was cannulated and blood samples collected before and after ACTH administration. Hypophysectomy by the trans-temporal approach had been done 14 weeks previously. The animal had grown very obese (gain of 5 pounds) in the postoperative period. This was presumed to be due to hypothalamic injury at the time of operation. The technique of the blood collection was the same as previously described. At the end of the experiment a complete autopsy was performed. A microscopic remnant of the anterior hypophysis estimated as one-fiftieth of the gland was detected in the examination of the serial sections of the sella. The cortex of both adrenals was about 2 mm. thick and showed a fairly uniform and

heavy disposition of lipids except for a lipid-poor subglomerular band. Within the reticular zone, which was rather broad, lipid was present in the form of giant droplets. The assay of the adrenal steroids is shown in Protocol III. The chemical assay was performed by Dr. Nelson.

These results show (a) that the concentration of these cortisone equivalents in the adrenal vein blood is approximately tenfold greater than that in peripheral venous blood, (b) that partial hypophysectomy (estimated as 98% complete) reduces considerably this output. They also suggest (c) that treatment with ACTH raises the level of cortisone equivalents as measured by the eosinophil response, and (d) ACTH administration to the hypophysectomized dog tends to bring this back to the normal level. In three

PROTOCOL III. DOG #46, FEMALE; HYPOPHYSECTOMY 14 WEEKS PREVIOUSLY; BODY WEIGHT 31 LBS.

Time	Blood flow, left adrenal vein ml./min.	No. mice given plasma extracts	Cortisone equivalents		
			Bioassay		Chemical assay
			Rate μg./min.	Concentration μg./100 ml. plasma	Concentration μg./100 ml. plasma
9:55–10:35	2.92	2	0.27	19 (27–11)*	40
	35 units ACTH in 120 ml. blood transfused during the next 80 minutes.				
10:35–11:55	0.92	2	0.38	71 (80–68)	180

* 68% confidence limits.

instances where a chemical assay was compared with the bioassay the former gave a value roughly twice that found by bioassay.

Assay of adrenal vein blood in cats.—Blood was collected from a vena caval pocket similar to that described by Stewart and Rogoff (1916). A polyethylene tube was inserted into the vena cava below the renal veins, with the tip of the tube at the level of the right adrenal. Ligatures were placed on all the veins contributing to that part of the vena cava above and below the adrenals, except the adrenal veins. The renal arteries and veins were tied off close to the kidneys. The upper vena caval ligature was the last one tied off and immediately after this, the blood flow into the cannula began. Blood collections were made for exactly 15 minutes. During this collection period saline was infused into a leg vein at the same rate as the flow of blood from the vena caval pocket. Three normal female cats were studied. The results are shown in Table 11. The values for the adrenal steroids appear to be lower than those found in the dogs that were studied.

Assay of adrenal steroids in human blood and urine.—In the previous section on *Parallelism of two curves* the procedure as applied to urine assay is

TABLE 11. ADRENAL STEROIDS IN THE ADRENAL VEIN BLOOD OF THE CAT

Cat no.	Blood flow	No. mice*	Cortisone equivalents	
			μg./min.	μg./100 ml. plasma
	ml./min.			
1	2.7	2	1.92	90 (92–87)†
2	3.4	2	1.06	46 (58–35)
3	2.33	2	1.32	99 (113–81)

* No. mice injected with plasma extract.
† 68% confidence limits.

described. In addition to the 4 assays mentioned in that connection, assays were done on the urine of 3 other normal human adults and on the urine of a patient with Cushing's disease.[7] In these latter assays the unextracted urine was injected at one dose level (2 ml.), the standard cortisone acetate at 2 dose levels; 42 mice were used for each assay. A chemical determination of the formaldehydogenic substance following the procedure outlined by Mason (1951) was done on each urine. In this method the urine was hydrolyzed at pH 1. A small aliquot of the urine from the patient with Cushing's disease was hydrolyzed with beta-glucuronidase and extracted according to the method of Dustan, Mason and Corcoran (1953). The extracted material was taken up into a 50% propylene glycol-water solution and injected into 6 mice. Eleven mice were injected with the standard. It is of interest to note (Table 12) that the urine from the patient with Cushing's disease showed a titer of adrenal steroids higher than normal by the bioassay method but not by the chemical method. These data suggest, also, that the conjugated adrenal steroids are biologically active since hydrolysis with beta-glucuronidase did not appear to alter the bioassay titer.

Blood from the patient with Cushing's disease and from a normal adult female were assayed for cortisone equivalents. The values are as follows:

	Sex	No. mice given plasma ext.	Cortisone equivalents
			μg./100 ml. plasma
Normal	F	2	11 (15–9)*
Cushing's disease	M	2	51 (73–29)

* 68% confidence limits.

The blood level of steroids was significantly higher in the patient with Cushing's disease.

[7] Patient under care of Dr. Louis Albert, Mt. Alto Hospital, U. S. Veterans' Administration, Washington, D. C.

TABLE 12. ASSAY OF CORTISONE EQUIVALENTS IN ADULT HUMAN URINE

Case No.	Sex	Volume Urine 24 hrs. ml.	Bioassay of Urine μg./24 hours	Chemical Assay—(formaldehydogenic) μg./24 hours
Normal #1	M	750	(a) 730 (1208– 398) (b) 650 (1125– 300) (c) 660 (1148– 315)	1080
#1		570	760 (1357– 331)	850
#2	M	1150	340 (506– 219)	1150
#3	F	2050	750 (1025– 513)	2580
#4	F	1560	730 (1108– 452)	1700
Cushing's Disease	M	2000	(d) 1800 (2280–1200) (e)* 1540 (2236–1064)	2100

* Assays (d) and (e) were done on aliquot samples. In assay (d) crude urine was injected; in assay (e) the extract of urine following hydrolysis with B glucuronidase was injected.

COMMENTS

Specificity

A satisfactory assay method should measure adrenal steroids whenever they are present in body fluids and should measure nothing else. With the present method substances in blood which affect eosinophils had a tenfold greater concentration in the adrenal vein than in the peripheral circulation and in a hypophysectomized dog the amount in the adrenal vein was very low compared to the normal but increased after ACTH administration. The titers obtained for blood and urine from a patient with Cushing's disease were elevated. These results, together with the original dose-response curve and the results obtained when cortisone was added directly to blood, show that when adrenal steroids were present, the bioassay method measured them.

Are there substances other than adrenal steroids in body fluids that the method will also measure? The parallelism of the curves for standard, urine and blood indicate that if there are such substances, they operate with essentially the same slope as cortisone acetate. Various sex hormones and non-adrenal steroids which might be present in urine or blood have been tested for eosinophil effects by Speirs and Meyer (1951) with negative or insignificantly low response.

Sensitivity

The eosinophils appear to respond to amounts of cortisone at least as low as 0.25 μg. For practical purposes we have taken 1 μg. as the minimum amount that can be easily assayed. In consequence we have been able to

assay crude urine directly, without extraction. The lowest amount detectable by liver glycogen methods in mice is 5 μg. of cortisone (Eggleston *et al.*, 1946), so that hydrolysis and extraction are required.

The sensitivity of the test varies with the strain of mouse. Rosemberg and Lewis (1950) using the eosinophil response in white mice reported that 20 μg. of cortisone was the minimum detectable amount. Speirs and Meyer (1951) using the Brown C-57 strain of mice reported a sensitivity of 0.5 μg. which is about the same level of sensitivity that we found.

Precision

The value of lambda (0.13) for assays of doses containing 10 μg. of cortisone equivalents is of the same magnitude as those reported by Venning *et al.* (1946) and by Dorfman (1950) in the liver glycogen assay, so that for these dose levels the two methods are equally precise. As the cortisone content of the unknown dose injected decreases below 10 μg. of cortisone, the present method loses precision, the liver glycogen methods lose sensitivity, i.e., the ability to assay at all. We are unable to make a similar comparison with the results of Speirs and Meyer (1951). The range of variation in response reported by them however appears much smaller than what we should expect on the basis of our own experience.

Blood titers

The blood and urine values presented in the present paper were obtained primarily to test the bioassay technique and not to establish normal values. For this latter purpose many more test subjects would be required. Comparisons of results with other methods may nevertheless be of interest.

By this bioassay method the cortisone equivalents per 100 ml. of plasma from peripheral blood of dogs was found to be 9–15 μg. After ACTH this was increased to 44 μg.; from peripheral blood of a normal woman it was 11 μg.; from adrenal vein blood of dogs it was 80–500 μg.; from peripheral venous blood of a patient with Cushing's disease it was 51 μg.; from adrenal vein blood of cats it was 80–100 μg. These bioassay values are consistent with the recent quantitative chemical determinations of adrenal steroids by Bush (1951, 1953), Sweat (1953), Farrell (1953), and Nelson and Samuels (1952). These investigators have used chromatographic procedures to purify their extracts. On the other hand the bioassay values are not consistent with chemical determinations upon extracts of blood without chromatography. Since chemical methods applied to mixtures of adrenal steroids measure biologically inactive steroids, such as tetrahydro E and F, as well as the major biologically active steroids, compounds F and cortisone, the bioassay method should and does give lower values with such mixtures than chemical methods do.

Urine titers

Most of the adrenal steroids in blood are unconjugated and readily extracted with organic solvents but in urine they are conjugated with glucuronic acid and not extractable. One problem, previously indeterminable, was the steroid content of the urine before hydrolysis. This bioassay method makes possible a determination of the cortisone equivalents of biologically active steroids by direct injection of the urine before hydrolysis or extraction. The high activity found in normal urine (340–760 μg. per 24 hrs.) and in the urine from a patient with Cushing's disease (1800 μg. per 24 hrs.) indicates that the conjugates are biologically active. When the latter urine was hydrolyzed with beta-glucuronidase, the titer was the same within the limits of error of the assays indicating the similarity in potency of the free and conjugated steroid.

SUMMARY

A bioassay method is presented for the determination of adrenal steroids in blood and urine using the eosinophil response of adrenalectomized mice. Appropriate statistical methods are developed. This method will routinely detect as little as 1 μg. of cortisone. This permits determination of adrenal steroids in urine without extraction. For doses containing 10 μg. the index of precision, λ is 0.13 ± 0.03; for those containing 1 μg., λ is 0.69 ± 0.04. Assays of blood and urine samples are presented.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Louis Alpert for permission to study his patient with Cushing's disease, Dr. Don H. Nelson for the chemical assays on blood, Mrs. Phoebe Prentiss for performing the formaldehydogenic assays in urine, and Mrs. Ina Hughes for assistance in the computations.

REFERENCES

BUSH, I. E.: *J. Physiol.* **115:** 12P. 1951.
BUSH, I. E.: *J. Endocrin.* **9:** 95. 1953.
DORFMAN, R. I.: *Ann. New York Acad. Sci.* **50:** 509. 1949.
DUSTAN H. P., H. L. MASON AND A. C. CORCORAN: *J. Clin. Investig.* **32:** 60. 1953.
EGGLESTON, N. M., B. J. JOHNSTON AND K. DOBRINER: *Endocrinology* **38:** 197. 1946.
FARRELL, G. L.: *Federation Proc.* **12:** 41. 1953.
FINNEY, D. J.: *J. Roy. Stat. Soc., Supp.* **7:** 155. 1941.
NELSON, D. H. AND L. T. SAMUELS: *J. Clin. Endocrinol. and Metab.* **12:** 519. 1952.
PERRY, W. L. M.: *Med. Res. Council, Special Report Series.* 270. 1950.
PORTER, C. C. AND R. H. SILBER: *J. Biol. Chem.* **185:** 201. 1950.
ROSEMBERG, E. AND R. A. LEWIS: *J. Applied Physiol.* **3:** 164. 1950.
SPEIRS, R. S. AND R. M. MEYER: *Endocrinology* **48:** 316. 1951.
STEWART, G. N. AND J. M. ROGOFF: *J. Pharmacol. and Exper. Therap.* **8:** 479. 1916.
SWEAT, M. L.: *Federation Proc.* **12:** 141. 1953.
VENNING, E. H. AND V. E. KAZMIN. *Endocrinology* **39:** 131. 1946.
VENNING, E. H.: *Methods in Medical Research*: **2:** 326. 1950.

THE EFFECTS OF CORTISONE ACETATE ON INTACT AND ADRENALECTOMIZED RATS DURING PREGNANCY[1]

M. EDWARD DAVIS AND ERNST JURGEN PLOTZ

Department of Obstetrics and Gynecology, the University of Chicago and The Chicago Lying-In Hospital, Chicago, Illinois

LEWIS (1923), Ingle and Fisher (1938) stated that after adrenalectomy in the rat gestation terminates in normal delivery. Other investigators found that removal of the adrenals resulted in abortion in some instances (Wyman, 1928; Dessau, 1937; Tobin, 1941). Britton and Kline (1936), McKeown and Spurrel (1940) reported that all adrenalectomized pregnant rats failed to go through the normal process of gestation. Attempts at replacement therapy by adrenal cortical extracts or hormones were mostly but not always successful (Britton and Kline, 1936; Dessau, 1937; Walaas and Walaas, 1944; Houssay, 1945). In view of these divergent results and the availability of crystalline cortisone, it became of interest to determine the effects of this hormone on intact and adrenalectomized pregnant rats.

MATERIAL AND METHODS

A total of 174 female rats of the Sprague-Dawley strain weighing between 210–250 gms. at the time of conception was used in the various experiments. The animals were kept in individual cages. They were fed "Rockland Rat Diet" ("complete") and allowed fluid ad libitum. In some groups tap water was replaced by 0.9% sodium chloride solution. The animals were observed closely and daily vaginal smears and body weights recorded. Evidence of abortion was ascertained by vaginal bleeding and autopsy findings.

Adrenalectomy according to the technique described by Richter (1941) was performed under ether anesthesia. Failure of complete adrenalectomy or the findings of accessory adrenal tissue occurred in a total of 13 rats. These animals were discarded. Following removal, the adrenals were dissected free of adhering fat, weighed immediately, and preserved in 10% formaldehyde. In those rats in which the gestation went to term the young were weighed and sacrified. The adrenals were removed and dissected cleanly. After fixation the fetal glands were dried completely and weighed. Representative glands were prepared for histological study, using Hematoxylin-Eosin or Sudan III.

The cortisone acetate[2] employed was a saline suspension of the micronized steroid. Injections were begun on the day of surgery and in the pregnant animals stopped on the day of parturition.

Received for publication August 8, 1953.

[1] This work was done under a grant from the Douglas Smith Foundation for Medical Research and the Joseph Bolivar DeLee Memorial Trust of the University of Chicago.

[2] Cortone (Merck).

The following groups of animals were studied. Twenty-one pregnant rats received no special treatment and served as controls. In a second group of 46 animals, 23 rats were adrenalectomized on the 4th–6th day, and 23 animals on the 14th–16th day of gestation. In 11 rats in each group the drinking water was replaced by 0.9 per cent saline solution; the remainder received no treatment except for the adrenalectomy.

A third group of 26 rats was adrenalectomized on the 4th–6th day of pregnancy (14 animals) and on the 14th–16th day (12 animals) and then treated with 2 mg. of cortisone acetate daily.

In 21 normal pregnant rats injections of 3 mg. cortisone acetate daily were started on the 4th–6th day and in 11 animals on the 14th–16th day of pregnancy. One mg. of cortisone acetate was given daily for varying periods (7–19 days) to 7 normal pregnant rats.

Sixteen non-pregnant female rats were adrenalectomized and used as controls to determine the survival time after operation.

RESULTS

The Effects of Adrenalectomy on Pregnant Rats

Outcome of pregnancy

When adrenalectomy was performed on the 4th-6th day of gestation all of the pregnancies terminated in abortions (Table 1). However, if the surgery was postponed until the 14th-16th day of gestation, with one exception all animals carried their pregnancies to term and delivered normally.

Survival time after operation

Pregnancy prolongs the survival time of rats adrenalectomized late in the gestation (Table 1). The animals which underwent surgery on the 4th-6th day of gestation survived for 18.5 days, while those from which the adrenals were removed on the 14th-16th day lived for 21 days. On the other hand, adrenalectomized non-pregnant animals died in 15.2 days. The dif-

TABLE 1. EFFECTS OF ADRENALECTOMY DURING PREGNANCY ON OUTCOME OF PREGNANCY AND SURVIVAL TIME AFTER OPERATION

Adrenalectomy	No.	Abortions	Survival time after operation in days	*P* versus non-pregnant controls
Non-pregnant rats	16	—	15.2 ($\pm$0.62)*	—
Pregnant rats, 1st half of pregnancy	12	12	18.5 ($\pm$0.50)*	>0.05
Pregnant rats, 2nd half of pregnancy	12	1	21.0 ($\pm$1.43)*	<0.01

* Calculation of standard error of the mean:

$$\text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

ference of 5.8 days between the non-pregnant controls and the animals operated late in pregnancy is statistically significant.

Body weight

It is well known that the weight of mothers increases progressively during pregnancy. When the adrenals were removed early in pregnancy and no further treatment was instituted the weight of the mothers declined continuously until death. Adrenalectomy late in pregnancy caused a decrease of the weight increment.

Control animals gained more than 50% in body weight during gestation. Rats which were adrenalectomized late in pregnancy increased only 25% in weight. Following parturition controls retained 15% of their weight gain while those adrenalectomized late in pregnancy retained only 0.3%.

Effect on litters

A significant difference in the size of litters and in the distribution of sex was not observed when the mothers were adrenalectomized late in pregnancy (Table 2). Adrenalectomy caused a higher incidence of stillborn and "sickly" young when the pregnancy was carried to term (14.4%) as compared with the incidence in the controls (2.7%). The term "sickly" is used for those newborn rats with poor vitality at birth. These animals were born alive but failed to survive.

Effect of fetal body weight and adrenals

A significant deficit in weight of the newborn rats was observed in the adrenalectomized mothers (Table 2). The average weight of the newborn in the control animals was 6.13 gm., in the adrenalectomized untreated animals 5.22 gm.

TABLE 2. EFFECTS OF ADRENALECTOMY ON LITTER, FETAL BODY WEIGHT AND ADRENAL WEIGHT

	Pregnant controls	Adrenalectomy 2nd half of pregnancy	*P* versus pregnant controls
No. of litters	21	11	—
Dead and "sickly" young	5	13	—
Vigorous young	182	78	<0.01
Fetal body weight (gm.)	6.13 (±0.10)*	5.22 (±0.30)*	<0.01
Fetal adrenal weight (mg.)	0.496 (±0.017)*	0.554 (±0.041)*	>0.3
$\frac{\text{Fetal body weight}}{\text{Fetal adrenal weight}} \times 1000$	12.35 (±0.34)*	9.42 (±0.31)*	<0.01

* Calculation of standard error of the mean:

$$\text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

The weight of the fetal adrenals was increased by adrenalectomy of the mother. In the non-treated adrenalectomized rats the average weight of the fetal adrenals was 0.554 mg., in the control animals 0.496 mg. The difference is not significant statistically, but the correlation of adrenal weight to body weight shows a statistically significant increase in adrenal weight of the newborn rats in the adrenalectomized group.

The enlarged adrenals of the fetuses of the untreated adrenalectomized mothers differ from the normal fetal adrenals at birth. There is a distinct tendency for an increase in the size of the three zones of the gland. The cells of the outer zones are enlarged. The central zone contains may distended blood sinusoids. An increased number of darkly stained cells in the central zone and in the other zones of the cortex which was reported by Walaas and Walaas (1944) was not obvious.

The Effects of Adrenalectomy on Pregnant Rats Maintained on Saline

Outcome of pregnancy

The beneficial effect of salt on the survival of adrenalectomized animals is well known. The replacement of tap water by 0.9 saline solution increased the number of pregnancies that carried to term. In 11 rats which were adrenalectomized on the 4th-6th day of gestation, 8 pregnancies were terminated successfully. In the group of 11 animals adrenalectomized on the 14th-16th day no abortions occurred.

Body weight

Treatment with physiological saline was of some benefit as far as weight gain of the mothers was concerned. Animals operated early gained 30.1% in weight, while those adrenalectomized late increased 35.2% in weight. Saline treatment resulted in a retention of 3% of weight in animals subjected to surgery early in pregnancy and 5.8% in those adrenalectomized late.

Effect on litters

A significant difference in the size of litters and in the distribution of sex was not observed in the adrenalectomized saline maintained mothers. The administration of saline solutions did not improve the vitality of the young of adrenalectomized mothers (Table 3).

Effect on fetal body weight and adrenals

The averge body weight of the newborn rats of the saline maintained mothers adrenalectomized early in pregnancy was 5.20 gm. The average weight of the newborn of saline treated animals operated on during the

TABLE 3. EFFECTS OF ADRENALECTOMY ON LITTER, FETAL BODY WEIGHT AND ADRENAL WEIGHT. MOTHERS MAINTAINED ON SALINE

	Adrenalectomy 1st half of pregnancy saline treatment	*P* versus pregnant controls	Adrenalectomy 2nd half of pregnancy saline treatment	*P* versus pregnant controls
No. of litters	8	—	11	—
Dead and "sickly" young	10		11	
Vigorous young	62	<0.01	84	0.028
Fetal body weight (gm.)	5.20 (±0.15)*	<0.01	5.81 (±0.16)*	>0.2
Fetal adrenal weight (mg.)	0.548 (±0.025)*	>0.1	0.615 (±0.028)*	<0.01
$\frac{\text{Fetal body weight}}{\text{Fetal adrenal weight}} \times 1000$	9.49 (±0.34)*	<0.01	9.45 (±0.29)*	<0.01

* Calculation of standard error of the mean:

$$\text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

second half of pregnancy, however, was 5.81 gm. and did not differ significantly from the control animals.

The weight of the fetal adrenals was increased when the mothers were adrenalectomized and maintained on saline. In the animals operated early the average weight of the fetal adrenals was 0.548 mg. while in those adrenalectomized late the fetal adrenals wighted 0.615 mg. The statistical proof for the absolute weight gain of the fetal adrenals was found in the group where the saline maintained mothers were adrenalectomized during the second half of pregnancy, but the correlation of body weight to adrenal weight showed a statistically significant increase in the adrenal weight in both groups.

The enlarged adrenals of the fetuses of the saline maintained adrenalectomized mothers resemble closely those of the fetuses of untreated adrenalectomizd mothers.

The Effects of Adrenalectomy on Pregnant Rats Treated with Cortisone Acetate

Outcome of Pregnancy

When 2 mg. of cortisone acetate was given daily after adrenalectomy interruption of pregnancy did not occur except in one case where the animal was adrenalectomized on the 4th day after mating.

Body weight

The administration of 2 mg. cortisone acetate after adrenalectomy did not restore the normal weight increase during pregnancy as observed in

untreated controls. The weight curve was similar to that obtained in the salt treated adrenalectomized animals. Rats operated early gained 30%, while those adrenalectomized late increased 28.8% in weight. Following parturition cortisone acetate treated adrenalectomized animals retained 2.1% and 7.5 % in weight, respectively.

Effect on litters

A significant difference in the size of litters and in the distribution of sex was not observed in the adrenalectomized animals treated with corti-

TABLE 4. EFFECTS OF ADRENALECTOMY ON LITTER, FETAL BODY WEIGHT AND ADRENAL WEIGHT. MOTHERS TREATED WITH 2 MG. CORTISONE ACETATE DAILY

	Adrenalectomy 1st half of pregnancy, 2 mg. cortisone acetate daily	*P* versus pregnant control	Adrenalectomy 2nd half of pregnancy, 2 mg. cortisone acetate daily	*P* versus pregnant controls
No. of litters	13	—	12	—
Dead and "sickly" young	43		18	
Vigorous young	64	<0.01	102	<0.01
Fetal body weight (gm.)	5.38 (±0.19)*	<0.01	5.19 (+0.15)*	<0.01
Fetal adrenal weight (mg.)	0.395 (±0.023)*	<0.01	0.373 (±0.019)*	<0.01
$\frac{\text{Fetal body weight}}{\text{Fetal adrenal weight}} \times 1000$	13.62 (±0.51)*	<0.01	13.19 (±0.23)*	<0.01

* Calculation of standard error of the mean:

$$\sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

sone acetate. Injection of 2 mg. cortisone acetate daily for 16–19 days caused a higher incidence of dead and "sickly" young (40.2%) than was observed in the control animals, in the untreated and saline maintained animals. However, only a slight but statistically significant increase in mortality of the newborn occurred (15.8%) when 2 mg. cortisone acetate was given for 6–9 days (Table 4).

Effect on fetal body weight and adrenals

Cortisone acetate administration to adrenalectomized pregnant rats did not produce any changes in the body weight of the fetuses as compared with the adrenalectomized untreated and saline maintained animals (Table 4).

A striking decreasing effect, however, was observed on the absolute and relative weight of the fetal adrenals when the mothers were treated with cortisone acetate after adrenalectomy. They averaged 0.395 mg. and 0.373 mg. in the cortisone acetate treated groups.

The Effects of Cortisone Acetate on Intact Pregnant Rats

Outcome of pregnancy

Three mg. cortisone acetate given daily in 33 intact pregnant rats for a period of 6–19 days did not cause interruption of pregnancy. All animals carried their pregnancies to term and delivered normally.

Body weight

The daily administration of 3 mg. cortisone acetate produced a striking inhibiting effect on the increase in weight of the mothers during pregnancy. The weight curve in these rats was very similar to the corresponding saline treated adrenalectomized animals and to the cortisone acetate treated adrenalectomized groups. No effect on weight gain was observed when only 1 mg. cortisone acetate daily was administered to normal pregnant rats.

Effects on litters

There was no significant difference in the number of young or in the distribution of sex in each litter in the cortisone acetate treated rats. No effect on the vitality was observed after 1 mg. cortisone acetate daily. When the daily dose was increased to 3 mg. there was an increased death rate of the newborn (Table 5). Injections of 3 mg. cortisone accetate daily for 16-19 days caused a high incidence of dead and sickly young (47.7%). When the same dose was given for 6–9 days a slight, but statistically significant increase of the mortality occurred (17.3%). The dead fetuses did not show signs of maceration in most cases. Death apparently occurred a short time before birth.

TABLE 5. INTACT PREGNANT RATS TREATED WITH 3 MG. CORTISONE ACETATE DAILY. EFFECTS ON LITTER, FETAL BODY WEIGHT AND ADRENAL WEIGHT

	Intact rats, 3 mg. cortisone acetate daily for 16–19 days	*P* versus pregnant controls	Intact rats, 3 mg. crotisone acetate daily for 6–9 days	*P* versus pregnant controls
No. of litters	21	—	11	—
Dead and "sickly" young	95		18	
Vigorous young	104	<0.01	87	<0.01
Fetal body weight (gm.)	6.18 (±0.10)*	>0.8	5.90 (±0.13)*	>0.1
Fetal adrenal weight (mg.)	0.386 (±0.015)*	<0.01	0.361 (±0.011)*	<0.01
$\frac{\text{Fetal body weight}}{\text{Fetal adrenal weight}} \times 1000$	16.01 (±0.52)*	<0.01	16.07 (±0.35)*	<0.01

* Calculation of standard error of the mean:

$$\text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

Effect on fetal body weight and adrenals

High dosage treatment (3 mg. daily) with cortisone acetate in the non-adrenalectomized pregnant rats did not affect the average body weight of the fetus when compared with the controls (Table 5).

The injection of 3 mg. cortisone acetate daily to intact animals definitely decreased the fetal adrenal weight (Table 5). They averaged 0.386 mg. and 0.361 mg. The relative decrease in weight of glands was smaller when 2 mg. cortisone acetate was given to adrenalectomized rats. The quotient body weight/adrenal weight ranged between 13.62 and 13.19 in these rats while the quotient averaged 16.01 and 16.07 in the cortisone acetate treated intact animals.

The adrenals of fetuses in which the mother was treated with 3 mg. cortisone acetate daily showed decrease in width of the cortex. The cells of the "intermediate zone" were tightly packed and smaller in size, especially in the periphery of this zone. The "subcapsular zone" remained unchanged. The sinusoids in the central zone seemed to be smaller than those in controls. The lipid content of the subcapsular zone was unchanged, but the outer part of the intermediate zone was considerably depleted of lipoids.

DISCUSSION

Pregnancy prolongs the survival time of adrenalectomized rats. It is generally assumed that this effect is due to the hormonal function of the corpus luteum, since pseudopregnancy prolongs the survival time in other species. Administration of progesterone has also been shown to maintain adrenalectomized animals in good health (Pfiffner *et al.*, 1934; Emery and Schwabe, 1936; Gaunt and Hays, 1938; Greene *et al.*, 1939; Collings, 1941). Intensive stimulation and luteinization of the ovaries by different gonadotropins increases the suvival period (Emery and Schwabe, 1936, in rats; D'Amour and D'Amour, 1939, in rats; Pfeiffer and Hooker, 1940, in mice). Dessau (1937) failed to obtain these results in adrenalectomized rats.

It has been suggested (Firor and Grollman, 1936; Billman and Engel, 1939) that the fetal adrenals provide the mothers with cortical hormones. In contrast to the report of McKeown and Spurrel (1940) who found a decrease in fetal adrenal weight, a significant increase was noted in our experiments, confirming the previous observations of Ingle and Fisher (1938), Walaas and Walaas (1944), and Houssay (1945). Walaas and Walaas stated that an increased number of darkly stained cells in the central zone in the enlarged fetal adrenals speak for an increased secretory activity. Their findings cannot be supported by the findings of this study. Tobin (1939) and Kitchell (1950) observed a compensatory hypertrophy of the remaining fetal adrenal when one was removed or destroyed during the last

week of pregnancy. This is a further evidence that the embryological adrenal is capable of responding to stimuli. However, it is not safe to conclude from the observations that the enlarged adrenals of the fetus can secrete sufficient amounts of cortical hormones to maintain the life of the mother during pregnancy.

Parturition is possible in the adrenalectomized rat when the operation is performed late in pregnancy. Saline solution (0.9%) instead of drinking water gives effective protection in many cases confirming the findings of McKeown and Spurrel (1940) and Houssay (1945). It has been demonstrated by various workers that extracts of adrenal cortex (Britton and Kline, 1936), desoxycorticosterone (Walaas and Walaas, 1944; Houssay, 1945) and cortin (Dessau, 1937) were all able to maintain gestation in adrenalectomized rats in most instances. Our own experiments show that adrenalectomized rats treated daily with 2 mg. cortisone acetate carried their pregnancies to term when surgery was performed early in pregnancy.

Robson and Sharaf (1951) reported interruption of pregnancy in 4 of 5 mice treated with 2 mg. cortisone for periods of 5 days. Glaubach *et al.* (1951) observed a lethal effect in utero to mice fetuses when 2.5 mg. cortisone acetate was injected 7–8 days before parturition. There was no interruption of pregnancy in intact rats when 3 mg. cortisone acetate daily was given in our own experiments. That amount is relatively smaller than that used by the other authors.

Houssay (1945) reported a prolongation of the gestation period after the administration of desoxycorticosterone to pregnant rats resulting in the excessive growth of fetuses and difficulties at parturition. In our experiments cortisone actate did not prolong the gestation time. Some rats had more bleeding than normally associated with prolonged parturition, but this was an exceptional complication.

Injections of 2 mg. cortisone acetate daily to adrenalectomized rats and 3 mg. cortisone acetate to intact animals was followed by a higher incidence of dead and sickly young. This toxic effect was not seen when a dosage of 1 mg. was used. LeRoy and Domm (1951) report death of fetuses which were injected subcutaneously with 500 μg. cortisone between the 11th-16th days of gestation. When Glaubach *et al.* (1951) administered 2.5 mg. cortisone acetate to mice 3–5 days before parturition the young were either dead or died shortly after birth. When this dosage was given 2 days before birth the mice were born alive but died within 2–5 days postpartum.

Treatment of the mothers with 3 mg. of cortisone acetate daily did not affect the size of the fetuses. In the adrenalectomized animals 2 mg. daily of cortisone acetate had no effect on the weight of the newborn. LeRoy and Domm (1951) made the same observation after subcutaneous injections

in rat fetuses. In decapitated fetuses no perceptible growth modifications were seen, when 250 gamma cortisone was given on the 16th day of uterine life. According to LeRoy and Domm the placental circulation accounts for the absence of the specific growth inhibiting effect of cortisone on the fetus (Wells and Kendall, 1940; Ingle, 1941). The growth retarding effect, however, can be observed in the chick embryo when cortisone is inoculated into the yolk sac (Karnofsky *et al.*, 1951).

The effect of cortisone acetate in causing a decrease in the size of the fetal adrenals was clearly seen in the young of adrenalectomized mothers treated with 2 mg. daily. It was also apparent in the offspring of intact animals that received 3 mg. of cortisone acetate daily.

While the growth inhibiting effect of cortisone acetate seems to be due to a direct effect on the body metabolism (Lewis *et al.*. 1950), it is generally assumed that the adrenal atrophy is produced entirely by a suppression of the secretion of the adrenocorticotrophic hormone. The source of this adrenocorticotrophic hormone is in doubt. It may have its origin in the maternal pituitary, the placenta, or in the fetal hypophysis. Houssay (1945) found that injections of ACTH to pregnant rats did not produce any enlargement of fetal adrenals. Greer (1949) did not find any evidence for adrenocorticotrophic activity of the placenta in hypophysectomized pregnant rats. It seems reasonable, therefore, to assume that the fetal ACTH production is suppressed by cortisone acetate administration. Several experiments substantiate this view. A decrease in adrenal size has been reported in decapitated fetuses of several species, in rats (Wells, 1948) in rabbits (Jost, 1948) and chicks (Case, 1951). This effect could be prevented by ACTH injections under the fetal skin (Wells, 1948).

The enlargement of the fetal adrenals in the adrenalectomized pregnant rats may then be explained on a similar basis. It can be assumed that the maternal cortical hormones have some inhibiting influence on the fetal pituitary. Adrenalectomy performed in the mother would consequently cause an increased release of the fetal ACTH.

The fetal adrenal in the adrenalectomized cortisone acetate treated rats (2 mg.) is larger relatively than the adrenal of offspring of intact mothers treated with 3 mg. of cortisone acetate daily. This can possibly be explained by the lower dosage or the absence of hormones produced by the maternal adrenal cortex influencing an increased release of fetal ACTH. It is rather questionable, however, whether the pituitary-adrenal system as found in the adult rat is completely identical with that of the fetus. Jailer (1950) found that in newborn rats injected with epinephrine or subjected to cold, the pituitary did not release ACTH until the 8th day and the 16th day, respectively, after birth.

SUMMARY

1. Pregnancy was interrupted in all cases when adrenalectomy was performed during the first week of pregnancy, but in only one instance when 12 rats were adrenalectomized during the third week of pregnancy. Only 3 cases of abortion were observed in 23 adrenalectomized rats maintained on salt. When 2 mg. cortisone acetate daily was given to 26 adrenalectomized rats, 25 rats carried their pregnancies to term. High dosage treatment (3 mg.) with cortisone acetate did not interfere with the outcome of pregnancy in any case.

2. A depression of the weight gain during pregnancy was found in all adrenalectomized rats as well as in the normal animals treated with 3 mg. cortisone acetate daily. No influence on the period of gestation was seen after cortisone acetate treatment.

3. The highest mortality rate of newborn rats was observed in the intact animals treated with 3 mg. cortisone acetate daily. In the adrenalectomized rats the mortality of those which were subjected to a cortisone acetate treatment (2 mg. daily) was significantly higher.

4. The growth retarding effect of cortisone did not influence the fetal weight.

5. A marked hypertrophy of the fetal adrenal was observed when the mothers were adrenalectomized. Saline treatment did not alter this observation.

6. A marked decrease in fetal adrenal weight occurred in all cortisone acetate (2 mg. and 3 mg.) treated animals. The most marked loss in fetal adrenal weight was observed when 3 mg. cortisone acetate was given to intact rats.

7. A dosage of 1 mg. cortisone acetate administered to intact rats did not influence the outcome of pregnancy, the weight gain during pregnancy, or the incidence of stillborns. There was no effect on the body weight or adrenal weight of the fetus.

REFERENCES

BILLMANN, F. AND R. ENGEL: *Klin. Wschr.* **18**: 599. 1939.
BRITTON, S. W. AND R. F. KLINE: *Am. J. Physiol.* **115**: 627. 1936.
CASE, J. F.: *Anat. Rec.* **109**: 277. 1951.
COLLINGS, W. D.: *Endocrinology* **28**: 75. 1941.
D'AMOUR, M. C. AND F. E. D'AMOUR: *Proc. Soc. Exper. Biol. & Med.* **40**: 417. 1939.
DESSAU, F.: *Acta brevia Neerl.* **7**: 55. 1937.
EMERY, F. E. AND E. L. SCHWABE: *Endocrinology* **20**: 550. 1936.
FIROR, W. M. AND A. GROLLMAN: *Am. J. Physiol.* **103**: 686. 1933.
GAUNT, R. AND H. W. HAYS: *Am. J. Physiol.* **124**: 767. 1938.
GLAUBACH, S., W. ANTOPOL AND S. GRAFF: *Bull. New York Acad. Med.* **27**: 398. 1951.
GREENE, R. R., J. A. WELLS AND A. C. IVY: *Proc. Soc. Exper. Biol. & Med.* **40**: 83. 1939.

GREER, M. A.: *Endocrinology* **45**: 178. 1949.
HOUSSAY, B. A.: *Rev. Soc. Argentina Biol.* **21**: 316. 1945.
INGLE, D. J. *Endocrinology* **29**: 649. 1941.
INGLE, D. J. AND G. T. FISHER: *Proc. Soc. Exper. Biol. & Med.* **39**: 149. 1938.
JAILER, J. W.: *Endocrinology* **46**: 420. 1950.
JOST, A.: *Compt. Rend. Soc. Biol.* **142**: 273. 1948.
KARNOFSKY, D. A., L. P. RIDGEWAY AND P. A. PATTERSON: *Endocrinology* **48**: 596. 1951.
KITCHELL, R. L.: *Proc. Soc. Exper. Biol. & Med.* **75**: 824. 1950.
LEROY, P. AND L. V. DOMM: *Anat. Rec.* **109**: 319. 1951.
LEWIS, J. T.: *Am. J. Physiol.* **64**: 503. 1923.
LEWIS, R. A., E. ROSENBERG AND L. WILKINS: *Endocrinology* **47**: 414. 1950.
MCKEOWN, T. AND W. R. SPURREL: *J. Physiol.* **98**: 255. 1940.
PFEIFFER, C. A. AND C. W. HOOKER: *Am. J. Physiol.* **131**: 441. 1940.
PFIFFNER, J. J., W. W. SWINGLE AND H. M. VARS: *J. Biol. Chem.* **104**: 701. 1934.
RICHTER, C. P.: *Endocrinology* **29**: 115. 1941.
ROBSON, J. M. AND A. A. SHARAF: *J. Physiol.* **114**: 11. 1951.
TOBIN, C. E.: *Endocrinology* **28**: 419. 1941.
TOBIN, C. E.: *Am. J. Anat.* **65**: 151. 1939.
WALAAS, E. and O. WALAAS: *Acta Path. Microbiol. Scandinav.* **21**: 640. 1944.
WELLS, B. B. and E. C. KENDALL: *Proc. Staff Meet. Mayo Clinic* **15**: 115, 324. 1940.
WELLS, L. J.: *Anat. Rec.* **103**: 563. 1949.
WELLS, L. J.: *Proc. Soc. Exper. Biol. & Med.* **68**: 487. 1948.
WYMAN, L. C.: *Am. J. Physiol.* **86**: 528. 1928.

COMPARATIVE EFFECTIVENESS OF ANTIHISTAMINES IN SUPPRESSION OF THE DECIDUAL CELL REACTION IN THE PSEUDOPREGNANT RAT

M. C. SHELESNYAK

Department of Experimental Biology, Weizmann Institute of Science, Rehovot, Israel

IN EXPERIMENTS undertaken to investigate the "non-specific" stimulus required to initiate the decidual cell reaction of the pseudopregnant uterus, it was demonstrated that topical application of diphenhydramine HCl (Shelesnyak, 1952a) and phenazoline HCl and promethazine HCL (Shelesnayak 1952b) inhibited the development of the deciduoma. Although these findings indicate a role of histamine release in the initiation of the decidual reaction, the lack of absolute specificity plus the existence of varied pharmacological actions of the antihistamines did not allow for a sharp definition of the mechanism of inhibition.

Of several approaches to the further exploration of the problem of deciduoma inhibition, two related studies were initiated, (1) the influence of various histamine antagonists with different activities and actions, and (2) the effect of various drugs with properties of certain antihistamines. This is a report of the relative effectiveness of seven histamine antagonists in the suppression of the decidual cell response of the pseudopregnant endometrium of the rat.

MATERIALS AND METHODS

The following antihistaminic agents were used: diphenhydramine HCl (*Benadryl*),* tripelennamine HCl (*Pyribenzamine*), pyranisamine maleate (*Neoantergan*),* phenazoline HCl (*Antistine*), prophen-pyridamine (*Trimeton*),* promethazine HCl (*Phenergan*)* and pyrathiazine ((*Pyrrolazote*).*

One hundred twenty adult female rats weighing from 160 to 200 grams were used. All animals had normal 4–5 day estrus cycles. To produce the required hormonal conditioning of the uterus, pseudo-pregnancy was induced by electrical stimulation of the cervical areas during proestrus (Shelesnyak 1931). On the fourth day of leucocyte vaginal smears, usually the fifth day after proestrus, the animals were operated under ether anesthesia, and both horns of the uterus exposed through a midventral abdominal

Received for publication August 8, 1953.

* The author gratefully acknowledges supplies of materials from the following: (1) Parke-Davis, U.S.A. for Benadryl, (2) Dr. D. Bovet, Instituto Superiore di Sanita, Rome for Neoantergan, Trimeton and Pyrrolazote, (3) Dr. B. Stein, Teva Chemicals, Jerusalem for Allersan, and (4) Dr. A. M. Davies, Israeli Inst. Biological Research for Phenergan made available to him by May & Baker, England.

incision. Both horns were irritated, either chemically by injection of 1% histamine dihydrochloride, or mechanically be scratching the antemesometrial wall with an 18 gauge hypodermic needle. Directly after irritation, the experimental horn was injected with 0.1 cc Ringer's solution containing the histamine antagonist under study; while the control horn was injected with 0.1 cc. Ringer's. This quantity of fluid filled the lumen and slightly distended the uterus.

Dose levels were established for each antihistaminic by using 5 rats per drug, one each receiving 0.1, 0.5, 1.0, 5.0 or 10.0 mg. Based on the results of this screening, closer graded doses were given in a second series. Twelve animals were used: 4 were given less than effective quantities, 4 partially suppressive doses, and 4 completely inhibitory doses. On the fourth day after the uterus was irritated and treated with antihistamine, the animals were sacrificed and the genital tract removed, mounted uniformly on cards to permit semiquantitative gross comparisons and fixed *in toto* for microscopic examination. Gross observations were adequate for study of comparative effects, and only random sections were made for microscopic study.

RESULTS

The results, tabulated in graphic form in Figure 1, reveal that all the antihistaminic agents studied suppressed decidual reactions, but not to the same degreee.

Histological examination revealed that effective inhibiting dose did not produce tissue damage which could be seen at the time of autopsy four days after application (Fig. 2). Moreover study of mitotic activity of the uterine mucosa showed no difference in degree of activity between experimentally inhibited horn and the developing deciduoma during the first 30 hours post-trauma, but after this time mitosis is no longer present in the suppressed side, but increases greatly in the deciduomata (Shelesnyak and Sachs).

The weakest inhibitor was prophenpyridamine (*Trimeton*). If *trimeton* inhibition is assigned a value of 1, then the order of inhibition-effectiveness of the other antihistamines is: pyranisamine maleate 2; tripelennamine 5; promethazine, diphenhydramine, and phenazoline 10; and pyrathiazine 25.

DISCUSSION

In earlier investigations attempting to explore the mechanism of blastocyst implantation, experiments undertaken to study the nature of the "non-specific" stimulus required to provoke the decidual reaction revealed that histamine antagonists inhibit the development of deciduoma (Shelesnyak, 1952a,b,c, 1953). Based on the general agreement that the deciduoma is comparable to the early normal maternal placenta (Seyle & McKeown 1935, Krehbiel 1937, Wislocki & Streeter, 1938, Nicholas 1942, 1947, Williams 1948, Swanberg 1950) it is considered that the exposition of the specific decidual cell inhibition may lead to the chemical agent involved in initiating the normal nidatory response of the progravid uterus.

Although antihistamine drugs suppress or inhibit most actions of his-

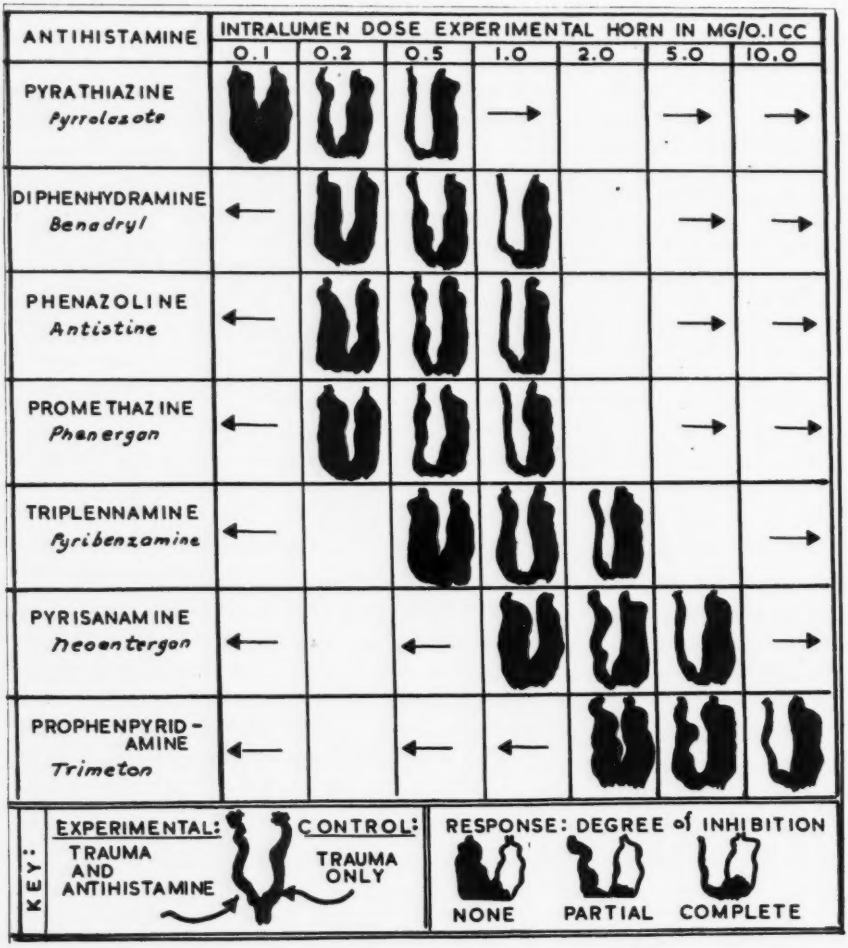


FIG. 1. Schematic representation of decidual growth and suppression.

tamine, it has been stressed by Lowe (1947) that "when antihistamine drugs, even though relatively specific are used as research tools or diagnostic aids, due consideration must be gven to a variety of pharmacological actions when experiments are planned and the findings interpreted." Briefly these pharmacological effects (other than specific histamine antagonism) include: atropine-like action, anti-spasmodic, spasmogenic, local anesthetic, enhanced pressor response to epinephrine, diminished pressor response to epinephrine, and adrenergic blocking. These properties are variously dis-

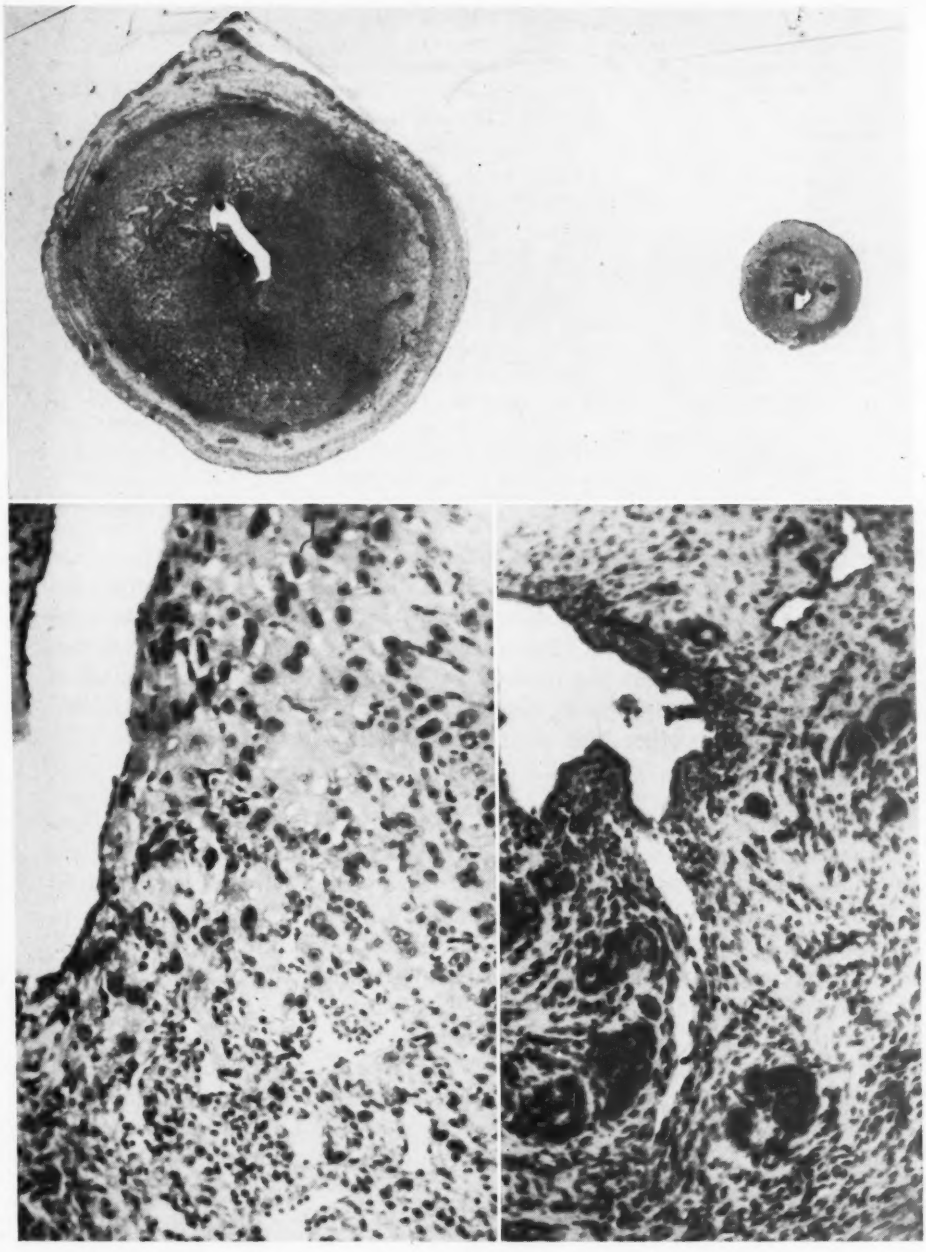


FIG. 2. Case. 58. Both horns of uterus traumatized, left side saline treated control shows large deciduoma after 96 hours, right side treated with 5 mgm. pyribenzamine at time of injury shows no decidual response. ×12 magnification. Lower photographs ×190 of deciduoma (left side) and suppressed horn (right side).

Drug action	Antihistaminic Drug							Reference
	Pyrathiazine	Diphenhydramine	Phenazoline	Promethazine	Tripelennamine	Pyranisamine	Prophenpyridamine	
Deciduoma Suppression	+++++	++++	++++	++++	+++	++	+	
Toxicity (Mouse)	+	++++	+++	+++	+++++	++++	++++	(1)
Antispasmodic (Guinea pig)	++++	+++	+	++++	++++	+++++	++	(1)
Iontophoretic (man)	?	+++++	+	?	++++	+++	++	(2)
Hayferer Alleviation	?	+++	++	++++	+++++	+	+++++	(3)
Uticaria Alleviation	?	++	+++	+++++	++++	+	+++++	(3)
Anesthetic	++++	+++	+++	+++	++++	++++	0	(4, 5, 6)
Atropine-like	+	+	±	?	±	±	?	(7)
Epinephrine Pressor-effect Enhancement	0	+++	+++	?	+++	+++	+++	(7)

(1) Leonard and Huttrer, 1950.
(2) Sternberg, *et al.*, 1950.
(3) Wishnefsky, 1950.
(4) Code *et al.*, 1950.
(5) Stephenson and Allmark, 1952.
(6) Loew, 1950 b.
(7) Loew, 1950 a.

FIG. 3. Semi-quantitative classification of various actions of seven antihistamines.

tributed among the different antihistamines. The findings of this investigation show that, though all the antihistamines tested inhibit the decidual response, no clear assignment of other pharmacological actions can be made on the basis of the results. Thus when comparison is made of relative effectiveness of the various pharmacological actions of these antihistamines to the deciduomata suppressing activity (Fig. 3) no singularly related pharmacologic action other than the antihistaminic effect appears.

SUMMARY

The comparative effectiveness of various antihistamines topically applied into the lumen of the irritated uterus of the pseudopregnant rat was studied in regard to the suppression of the decidual cell reaction. Pyrathiazine (*Pyrrolazote*) was effective in suppressing the deciduoma when applied in a dose of 0.2 mg. Diphenhydramine (*Benadryl*), phenazoline (*Antistine*) and promethazine (*Phenergan*) inhibited deciduomata formation in doses of 0.5–1.0 mg.; pyranisamine (Neoantergan) in 2–5 mg.; and prophenpyridamine (*Trimeton*) in 5–10 mg. doses.

When rank order of effectiveness of various pharmacological actions of these drugs is compared with effectiveness in respect to deciduoma suppression, no clear assignment of drug action beyond the antihistaminic activity can be made for the suppression of decidual development.

REFERENCES

CODE, C. F., J. U. KEATING AND M. D. LEAVITT, JR.: *Ann. N.Y. Acad. Sci.* **50**: 1177. 1950.

KREHBIEL, R. H.: *Physiol. Zool.* **10**: 212. 1937.

LEONARD, F. AND C. P. HUTTRER: *Histamine Antagonists.* Washington, NRC. 1950.
LOEW, E. R.: *Ann. N.Y. Acad. Sci.* **50:** 1142. 1950a.
LOEW, E. R.: *Med. Clinics N.A.* **34:** 351. 1950b.
NICHOLAS, J. S.: *Anat. Rec.* **83:** 457. 1942.
NICHOLAS, J. S.: *Quart. Rev. Biol.* **22:** 179. 1947.
SEYLE, H. AND T. MCKEOWN: *Proc. Roy. Soc. London, s.B.* **119:** 1. 1935.
SHELESNYAK, M. C.: *Anat. Rec.* **49:** 179. 1931.
SHELESNYAK, M. C.: *Bull. Res. Council Israel* **2:** 74. 1952a.
SHELESNYAK, M. C.: *Bull. Res. Council Israel* **2:** 202. 1952b.
SHELESNYAK, M. C.: *Am. J. Physiol.* **170:** 522. 1952c.
SHELESNYAK, M. C.: *Bull. Res. Council Israel* **3:** 112. 1953.
STEPHENSON, E. H. AND M. G. ALLMARK: *Rev. Canadienne d. Biol.* **11:** 93. 1952.
STERNBERG, T. H., D. J. PERRY AND P. LEVAN: *J.A.M.A.* **142:** 969. 1950.
SWANBERG, H.: *Acta Physiol. Scandinav.* **23:** Suppl. 79. 1950.
WILLIAMS, M. F.: *Am. J. Anat.* **83:** 247. 1948.
WISHNEFSKY, N.: *Antihistamines, Industry & Product Survey,* Chemonomics, 1950.
WISLOCKI, G. B. AND G. L. STREETER.: *Contr. Embryol.* Carnegie Inst. **27:** 1. 1938.

MAINTENANCE OF BLOOD 17-HYDROXYCORTICOSTEROIDS FOLLOWING ASCORBIC ACID TREATMENT

HABEEB BACCHUS

Research Laboratory, Department of Physiology, The George Washington University School of Medicine, Washington, D. C.

IT HAS been demonstrated that the eosinopenic and lymphopenic actions of exogenous and endogenous adrenal cortical hormones are prolonged by ascorbic acid treatment (Bacchus *et al.*, 1952). Those data were interpreted as indicating that ascorbic acid in some manner maintains the circulating levels of these hormones. It was observed, also, that simultaneous ascorbic acid treatment to adrenalectomized female rats receiving cortisone prevented the usual rise in the urinary 17-ketosteroids, and permitted a significant increase in the corticosteroid excretion (Bacchus, Altszuler, and Heiffer, 1952a; Bacchus and Heiffer, 1953). These data were also supported by *in vitro* studies which indicated that ascorbic acid depresses the metabolism of cortical hormones by liver slices (Bacchus, 1953 a, b).

This paper reports data on the effect of simultaneous ascorbic acid treatment on the circulating levels of 17-hydroxycorticosteroids in adrenalectomized rats injected with cortisone acetate. Data on the circulating leukocytes are also presented.

MATERIALS AND METHODS

The first experiment involved the determination of plasma 17-OH corticosteroids in adrenalectomized rats injected with cortisone acetate, with and without, ascorbic acid. For collection of blood, the animals were anesthetized with nembutal, laparotomy was performed, and blood collected by syringe from the abdominal aorta. The blood was placed in oxalated tubes, then centrifuged for five minutes. The plasma was then aspirated by syringe and the volume measured. The plasma of 3–5 animals was pooled to make a total of 15–25 ml. The plasma was then extracted with three volumes of chloroform. The extracts were evaporated to dryness in front of a fan. The extracts were taken up in 5 ml. chloroform and applied to magnesium trisilicate-Celite columns as used by Nelson and Samuels (1952).

Chromatography.—Magnesium trisilicate (one part) and Celite analytical filter-aid (one part) were mixed, washed in absolute ethanol, dried, and baked, as recommended by Nelson and Samuels (1952). The columns were prepared as recommended by those workers, 1.0–1.5 gm. of the magnesium trisilicate-Celite mixture were mixed with fifteen

Received for publication August 10, 1953.

ml. of chloroform and poured into the columns. After passage of an additional 10 ml. of chloroform through the columns, the crude extracts were introduced in 5 ml. chloroform. Twenty-five ml. of 2% ethanol in chloroform was passed through the column and discarded. Twenty-five ml. of 15% ethanol in chloroform was then passed through the column and collected in one vessel (eluate (a)). An additional twenty-five ml. of 15% ethanol in chloroform was passed through the column and also collected (eluate (b)). These eluates were then divided into two equal parts, viz., 1a and 2a, and, 1b and 2b, respectively. These aliquots were evaporated to dryness in a vacuum desiccator under regulated vacuum. The evaporated aliquots were each dissolved in 0.4 ml. of methanol and used for the Porter-Silber assay (1950).

Analytical procedure.—The method employed in this study was modified from Porter and Silber (1950) and from Nelson and Samuels (1952). The Nelson and Samuels micro-modification of the Porter Silber assay involved the use of final volumes of 0.5 ml. which were placed in appropriate cuvettes and read on the Beckmann spectrophotometer. In the present study the final volume was 1.0 ml., and the optical densities of the solutions were read on the Coleman Junior spectrophotometer with the appropriate adapter. It was found advisable in this study to employ larger (15–25 ml.) amounts of plasma in order to obtain accurate and reliable results. The dilute H_2SO_4, and the phenylhydrazine hydrochloride in dilute H_2SO_4 were prepared according to Nelson and Samuels (1952). The evaporated aliquots of the eluates were dissolved in 0.4 ml. of methanol, and to these were added 0.6 ml. amounts of phenylhydrazine-H_2SO_4, or of H_2SO_4. The phenylhydrazine-H_2SO_4 was added to aliquots 1a and 1b, while H_2SO_4 was added to aliquots 2a and 2b, respectively. These were mixed well, then incubated at 60° C. for 1 hour. After cooling in running tap water for 3 minutes, the solutions were poured into small cuvettes (10×75 mm.) and the optical densities read on the Coleman Junior spectrophotometer at 410 mμ. These optical density readings were used for the calculation of the corrected optical densities, i.e.,

$$\text{Corrected O.D.} = (\text{O.D.}_{1a} - \text{O.D.}_{2a}) - (\text{O.D.}_{1b} - \text{O.D.}_{2b})$$

These corrected optical densities were compared to corrected optical densities of known amounts of cortisone (O.D. of standard in phenylhydrazine-H_2SO_4 − O.D. of standard in H_2SO_4), and the amounts in the individual extracts calculated. Nelson and Samuels (1952) employed the empirical correction factor (absorption factor) recommended by Allen (1950) in preference to the controls recommended by Porter and Silber (1950).

CONTROL EXPERIMENTS

1. Experiments were designed to test the reliability of the chromatography columns used in this study. In initial experiments large amounts of cortisone and of desoxycorticosterone were introduced into the columns and eluted as above. The macro-Porter-Silber assay method was used to determine the recovery of cortisone, and the Heard-Sobel procedure (1946) was used to determine the recovery of DOC. Both of these methods have been used extensively in this laboratory and were observed to be highly reliable. The data indicated that 95–112% of the steroids were recovered from these columns. Small amounts of cortisone were also introduced into such columns, and the recovery determined by the semi-micro-Porter-

Silber method as described above. It was observed that the recovery of small amounts of cortisone ranged from 92% to 110%.

2. Experiments on the analytical procedure: Known amounts of cortisone were used for color development as described above; the method revealed a 4% variation in the optical density readings. Studies with amounts of cortisone ranging from 1 μg. to 20 μg. revealed that the color development was in accordance with Beer's Law. The recovery of known amounts of cortisone from the chromatography columns was checked several times during the progress of the experimental work. Since these tests indicated a mean recovery of about 95% no correction of the final values in the experimental series was warranted. While the present work was in progress Bayliss and Steinbeck (1953) published a modification of the Porter-Silber assay applicable to plasma extracts. Their method was essentially similar to that of Nelson and Samuels with the following exceptions: (a) the empirical absorption factor was not employed, the original chemical "blanks" as recommended by Porter and Silber (1950) (as used in this study) were employed. The recovery values were reported to be higher by this method. (b) Bayliss and Steinbeck corrected the values for efficiency of the chromatography columns. As stated above, the recoveries from the columns used in the present study were uniformly good, hence we did not consider any correction necessary.

EXPERIMENTS AND RESULTS

Adrenalectomized male rats (200–300 gm.) were used in this experiment. The animals were bilaterally adrenalectomized under ether anesthesia at least 2 days before experimentation, and were subsequently maintained on an *ad libitum* fare of Purina Laboratory Chow and 0.9% NaCl solution. Group A animals were given single subcutaneous injections of cortisone acetate (2.0 mg./100 gm.) along with intraperitoneal injections of ascorbate (ascorbic acid injectable, Roche, Na ascorbate) at the rate of 10 mg./100 gm. Group B consisted of adrenalectomized rats which received cortisone acetate as above, but which received intraperitoneal injections of saline instead of ascorbate. Groups of 3–5 animals per experimental group were killed at the following times: before injections, 3 hr., 24 hr., 48 hr., 72 hr., and 96 hr., after the injections. In addition some animals were given ascorbate alone and killed 3 to 4 hours after. The animals were anesthetized with nembutal, laparotomy was performed, and blood was collected from the aorta. The plasma content of 17-OH corticosteroids was determined.

The mean values and the standard errors were calculated from the *determined* values, the pooled plasma from 3–5 animals contributing to one

determined value. The standard error was calculated in the following manner:

$$\text{S.E.} = \sqrt{\frac{\sum(x-\bar{x})^2}{N(N-1)}}$$

in which N is equal to the number of *determined* values. The data are presented in Figure 1. No 17-OH corticosteroids were detectable in the plasma of the animals which received no cortisone. Ascorbate treatment

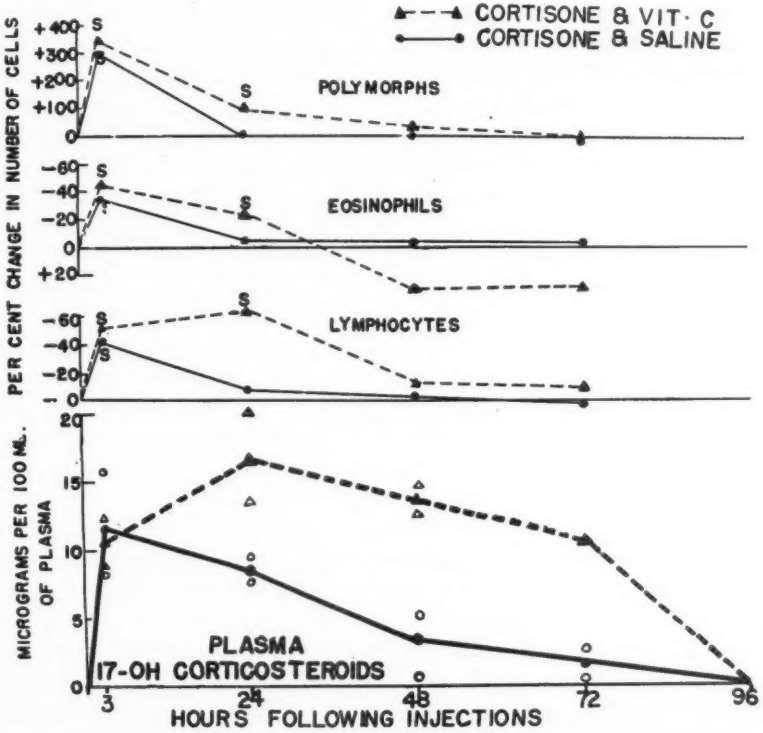


FIG. 1. Plasma 17-OH corticosteroids, and blood leukocytes in adrenalectomized rats treated with cortisone acetate and ascorbate, or with cortisone acetate and saline. Groups of animals were anesthetized at the intervals indicated, and blood obtained for 17-OH corticosteroid determinations. Statistical treatment of the data on plasma corticosteroids was described in text. S.E. = open symbols. Hematologic data were obtained from separate groups of animals. "S" indicates that the mean difference from the count at 0 hour is statistically significant. The circulating numbers of cells in each animal, at each instant, were compared to the numbers of 0 hour, and the mean differences $\pm$S.E. were calculated. Per cent changes are presented in the figure for the hematologic data.

to such animals also failed to result in the appearance of detectable amounts of steroids in the plasma. Three hours after the injections of cortisone, detectable amounts of 17-OH corticosteroids were observed in the plasma of both groups of animals. Three determined values per group (from 9 animals per group), at this time showed no difference between the levels in the two groups. Twelve rats per group, contributing to 3 determined values per group, were used for the determination of the circulating 17-OH corticosteroids at the end of 24 hours after the injections. The content in the plasma of animals of group A was significantly greater ($P < 0.05$) than that in Group B animals. Eleven animals per Group (three determined values per group) were used for the 48 hour determinations. At this time the content in the Group A animals was significantly greater than that in the Group B animals. Four animals of Group A (one determination), and six animals of Group B (two determinations) were used 72 hours after the injections. Since only one determined value was obtained from the group A animals statistical comparison at this time was not possible. The value in the Group A animals was well out of the range in the Group B, however. No corticosteroids were present in either group at 96 hours.

The above data indicate that ascorbate treatment into cortisone-treated adrenalectomized rats results in a maintenance of the circulating level of 17-OH corticosteroids.

Hematologic studies.—Two groups of 6 adrenalectomized rats each were treated as the Groups above. Total leukocyte counts, lymph. : polymorph ratios, and total esoinophil counts, were made by Randolph's (1944) method at 0, 3, 24, 48 and 72 hours, after the injections. Figure 1 presents data indicating that ascorbate treatment is associated with a prolongation of the hematologic effects of cortical hormones. In the present study, in which 10 mg./100 gm. was the dosage of ascorbate used, the hematologic actions of the injected cortisone acetate are prolonged to 24 hours. At the end of 48 hours the hematologic pattern has returned to pre-injection levels, essentially. In the animals which received cortisone and saline, the hematologic effects of the injected cortisone acetate disappeared by the end of 24 hours after the injections. The data of this experiment indicate that the blood elements provide a peripheral indication of persistent levels of 17-OH corticosteroids in Group A rats at 24 hours following the injections. There is a disappearance of these changes at the subsequent 24-hour intervals.

DISCUSSION

In a previous study it was shown that 100 mg. of ascorbate per 100 gm. given along with 2 mg. of cortisone prolonged the eosinopenic and lymphopenic actions of cortisone for 24 hours. No data were provided on the level

of circulating cortisone as detected by chemical methods. In the present study a similar prolongation of the hematologic effects of cortisone is noted when 10 mg. of ascorbate is used. It has been observed in other work that 1.0 mg. of ascorbate is also capable of prolonging the action of the injected cortisone. The hematologic data indicate that the prolongation lasts only 24 hours, there occurring a reversal by 48 hours.

The chemical data reveal that the circulating cortisone is probably maintained for a longer time than is indicated by the hematologic data, in the animals which received ascorbate. These data are in consonance with our previous observation (Bacchus and Heiffer, 1953) that the increased excretion of corticosteroids in animals receiving ascorbate and cortisone continued for 3 post-injection days. The increased corticosteroid excretion was interpreted as indicating that there was a plethora of circulating cortical hormone. This is further substantiated by the observation that ascorbate is capable of depressing the urinary 17-ketosteroids without altering the urinary corticosteroids in animals which received 1 mg. of cortisone instead of 2 mg. *In vitro* data from this laboratory provide indirect support for the above observations. It was observed that ascorbate is capable of suppressing the *in vitro* metabolism of adrenal steroids by liver slices (Bacchus, 1953 a, b).

The reason for the failure of the hematologic response after 24 hours is not apparent. It is quite likely, however, that the change in response indicates a refractoriness of the target organs involved. It is well known that the hematologic criteria of adrenal function are most reliable in acute situations. These data further emphasize that the hematologic criteria of corticosteroid titers may be unreliable in chronic conditions.

The data of this study suggest that great care should be taken in evaluating and analyzing data obtained following ascorbic acid treatment. We had shown that the urinary changes in steroid excretion following ingestion of ascorbic acid by intact human subjects (Kayahan, 1952) may not be ascribable to changes in adrenal function(s). Similar urinary changes had been observed in adrenalectomized female rats given cortical hormone and ascorbate (Bacchus and Heiffer, 1953), hence the changes are probably related to the peripheral metabolism of the steroids, and to the action of ascorbate on this process.

SUMMARY

The circulating levels of 17-OH corticosteroids in adrenalectomized male rats injected with cortisone and ascorbate were studied. The data revealed that ascorbate is capable of maintaining the circulating titer of 17-OH corticosteroids for 72 hours after the injection. Hematologic data revealed peripheral indication of a persistent high level of the hormone up

to 24 hours after the injection, following which time the hematologic changes disappeared. The latter observation suggests a refractoriness of the target organs involved to the hormone in circulation. The data are discussed in relation to our *in vivo* and *in vitro* observations which suggest that ascorbate may depress the metabolism of cortical hormones.

REFERENCES

ALLEN, W. M.: *J. Clin. Endocrinol.* **10**: 71. 1950.

BACCHUS, H., N. ALTSZULER AND M. H. HEIFFER: *Endocrinology* **51**: 302. 1952.

BACCHUS, H. AND M. H. HEIFFER: *Am. J. Physiol.* **173**: 33. 1953.

BACCHUS, H., N. ALTSZULER AND M. H. HEIFFER: *Proc. Soc. Exper. Biol. and Med.* **80**: 88. 1952.

BACCHUS, H.: *Endocrinology.* **53**: 441. 1953a.

BACCHUS, H.: *Endocrinology* **53**: 617. 1953b.

BAYLISS, R. I. S. AND A. W. STEINBECK: *Biochem. J.* **54**: 523. 1953.

HEARD, R. D. H. AND H. SOBEL: *J. Biol. Chem.* **165**: 687. 1946.

KAYAHAN, S.: *J. Endocrinol.* **8**: 211. 1952.

NELSON, D. H. AND L. T. SAMUELS: *J. Clin. Endocrinol. and Metab.* **11**: 519. 1952.

PORTER, C. C. AND R. H. SILBER: *J. Biol. Chem.* **185**: 201. 1950.

RANDOLPH, T. G. *J. Allergy* **15**: 89. 1944.

INFLUENCE OF A POTENTIATOR OF ESTROGEN IN THE INHIBITION OF PROGESTERONE IN DEVELOPMENT OF DECIDUOMATA[1]

FREDERICK L. HISAW AND JOSEPH T. VELARDO[2]

The Biological Laboratories, Harvard University, Cambridge, Massachusetts

COHEN and Bates (1952) reported that the non-ketonic neutral material remaining in the toluene fraction obtained from the phenolic extract of the urine of pregnant mares contained a non-estrogenic substance that would augment the action of estrone and diethylstilbestrol on the vaginal mucosa and uterus of the castrated rat. Although this "neutral" fraction did not produce vaginal cornification it did, at high dosage, cause some increase in weight of the uterus in castrated rats that had been hypophysectomized or adrenalectomized. Since estrogens antagonize the action of progesterone on decidual development (see review by Courrier, 1950) and such inhibition is directly proportional to estrogenic potency (Velardo and Hisaw, 1951) it seemed of interest to determine the effects this augmentor[3] may have under these conditions.

PROCEDURES

The procedures used in these experiments were essentially those previously described (Velardo and Hisaw, 1951; and Hisaw and Velardo, 1951). It may be stated briefly that pseudopregnancy was induced in rats by electrical stimulation of the uterine cervix. The animals were castrated on the fourth day of pseudopregnancy and the right horn of the uterus was traumatized throughout its entire length by scratching the antimesometrial surface of the endometrium. A daily dose of 1.5 mg. progesterone was injected subcutaneously for three days. This dosage was sufficient to elicit a decidual reaction comparable to that found in normal pseudopregnant rats 72 hours after uterine traumatization. The daily injections of estrogens and the augmenting substance were given concurrently with the progesterone and were administered subcutaneously in sesame oil at separate sites. Seventy-two hours after the initial injection, the rats were killed, and the uteri weighed on a Roller-Smith torsion balance.

RESULTS

The results (Table 1) clearly indicate that three daily injections of 54 μg. of the augmentor alone do not appreciably modify the weights of either

Received for publication August 18, 1953.

[1] This investigation was supported in part by a research grant from the National Institutes of Health, U.S. Public Health Service.

[2] Present address: Department of Pathology, Harvard Medical School, Boston 15, Massachusetts.

[3] The term augmentor has been given to this material by Cohen and Bates (1952).

the traumatized or non-traumatized horns of ovariectomized, pseudopregnant rats. There is no statistical difference between animals given the augmentor and the non-treated controls in the weight of the uterus or in the percentage increase of the traumatized horn over that of the non-traumatized contralateral horn. Nor was there a noticeable difference in the morphology of the uteri and vaginae. The reproductive tracts of both groups showed conditions typical in most respects of those found on the third day following castration.

It also is clearly indicated (Table 1) that the augmentor did not influence the action of progesterone in the development of deciduomata. There is no statistical difference between the weights of traumatized uteri of castrated psuedopregnant rats given graded doses of progesterone in combination with the augmentor and those of animals on comparable doses of progesterone alone. However, the only definite action known for the augmentor is that of enhancing the effectiveness of estrogens in promoting growth of the uterus, and for this study we were primarily interested in the possible influence this might have on the ability of estrogens to inhib t progesterone in the development of deciduomata.

We have reported that the decidual reaction induced in the uteri of castrated, pseudopregnant rats by three daily injections of 1.5 mg. progesterone is decreased by about one-half by concurrent injections of 0.1 μg. estradiol-17β, 0.3 μg. estradiol benzoate, 0.6 μg. diethylstilbestorl, 1.6 μg. estrone, or 20 μg. estriol (Velardo and Hisaw, 1951; and Frieden and Velardo, 1952). These observations show that the ability of an estrogen to inhibit progesterone under these conditions is directly proportional to its estrogenic potency. Therefore, if the augmentor increases the effectiveness of an estrogen this should be reflected in a corresponding decrease in the decidual reaction. This in general is true. When 1.5 mg. progesterone is unopposed the average weight of the decidual reaction in the traumatized horn is 545.7 mg. (Table 1) and this is reduced to 333.4 mg. when 0.1 μg. of estradiol is added to the treatment. The data also show that a further decrease in weight of the traumatized horn occurs when 1.0 μg. of the augmentor is given concurrently with 1.5 mg. progesterone and 0.1 μg. estradiol (280.0 mg.) and that increases in dosage of augmentor intensify the inhibition. Since the average weight of deciduomata produced by 1.5 mg. progesterone in combination with as much as 54 μg. of the augmentor is 510 mg. it is concluded that the observed increases in inhibition are due to a potentiation of estrogen. The data recorded in Table 1 show that these observations for estradiol also hold for estrone and estriol, but apparently do not apply to estradiol benzoate and diethylstilbestrol.

A point of considerable interest is brought out in these experiments

TABLE 1. ACTION OF AUGMENTOR ON ESTROGEN INHIBITION OF DECIDUAL DEVELOPMENT

No. of rats	μg. estrogen daily dose	μg. augmentor daily dose	Traumatized horn, in mg.	Control horn, in mg.	% increase[1] over control horn
5	0.0	0.0[2]	152.2±10.6[3]	127.2± 8.1	20.0± 2.0
5	0.0	0.0; 0.5 mg. Prog.	280.5± 6.2	100.4± 5.2	180.0±10.1
5	0.0	0.0; 1 mg. Prog.	437.1±17.3	106.9± 4.7	307.1±21.2
37	0.0	0.0	545.7±20.5	120.5± 6.5	357.0±35.0
5	0.0	1.0[2]	147.5±11.3	125.8± 7.0	16.4± 3.5
5	0.0	54.0[2]	168.3±11.7	130.0± 7.1	29.8± 3.1
4	0.0	54.0; 0.5 mg. Prog.	300.0± 9.1	118.1± 8.8	163.0± 8.5
5	0.0	54.0	510.1±18.3	125.0± 7.4	320.0±27.1
15	0.1 Est. 17β	0.0	333.4±14.5	122.1± 9.2	178.0±15.4
4	0.1 Est. 17β	0.5	302.1±10.6	120.1±10.8	157.1±13.6
7	0.1 Est. 17β	1.0	280.0±13.8	195.0±10.0	49.0± 8.1
9	0.1 Est. 17β	5.0	262.1±14.4	176.4± 6.1	47.4± 4.0
9	0.1 Est. 17β	10.0	256.4±11.1	177.7± 5.8	41.0± 6.3
5	0.3 Est. benz.	0.0	317.6± 8.2	117.2± 5.0	172.0±12.2
6	0.3 Est. benz.	1.0	299.0±10.7	162.1± 7.1	83.0± 9.1
6	0.3 Est. benz.	10.0	298.1±11.1	161.0± 9.7	85.1± 7.9
4	0.3 Est. benz.	15.0	295.2±17.4	154.4±13.2	91.5±10.0
6	0.3 Est. benz.	20.0	290.8±20.2	160.0±14.6	81.9± 8.7
5	0.6 Diethylstilbestrol	0.0	284.0±11.5	105.4± 8.6	171.0±16.3
4	0.6 Diethylstilbestrol	1.0	297.7± 6.7	156.1±10.5	90.5± 4.8
4	0.6 Diethylstilbestrol	10.0	313.1±10.6	174.7±11.7	78.4±10.4
4	0.6 Diethylstilbestrol	15.0	288.2±17.1	164.4± 9.1	74.1±13.0
5	1.6 Estrone	0.0	336.1±18.5	132.0±11.3	171.0±15.1
5	1.6 Estrone	1.0	337.0±16.9	156.0±10.5	108.0±11.7
5	1.6 Estrone	10.0	273.2±13.9	161.4± 6.0	71.0±15.4
4	1.6 Estrone	15.0	242.5±11.6	145.7± 9.6	67.3±11.1
4	1.6 Estrone	20.0	245.9±18.2	164.0±10.0	49.9± 8.8
5	20.0 Estriol	0.0	281.0±12.4	100.1± 6.3	181.0±12.1
5	20.0 Estriol	1.0	270.0±15.5	121.0±11.4	122.0±10.4
4	20.0 Estriol	10.0	229.0±11.8	151.0±10.0	43.0± 8.7
3	20.0 Estriol	15.0	254.1±18.9	154.0±14.6	65.0±10.9
4	20.0 Estriol	20.0	241.0±16.3	154.0±13.1	56.9± 7.2

[1] $\frac{[\text{Wt. traum. horn}) - (\text{Wt. control horn})]}{(\text{Wt. of control horn})} \times 100.$

[2] These animals received no progesterone; all others received 1.5 mg. progesterone daily for three days unless otherwise indicated.

[3] Avg. ± Standard Error.

when comparisons are made of the effects of the various treatments on the control, non-traumatized horn (Table 1). In rats castrated on the fourth day of pseudopregnancy and given no hormonal treatment, the non-traumatized uteri had an average weight of 127 mg. when the animals were killed at the conclusion of the three-day experimental period. Comparable animals given 0.5, 1.0, or 1.5 mg. progesterone daily for the three-day period had uteri that weighed less than those of the non-treated castrates while the highest dosage of the augmentor used (54 μg.) produced no significant effect on uterine weight (130 mg.). Nor was there an increase in weight of the non-traumatized horn when progesterone and the augmentor were given together. Thus, it was found that the augmentor, at the dosages employed, did not stimulate growth of the non-traumatized horn of the uterus either when given alone or in combination with progesterone.

However, the results were quite different when estrogens were included in the treatments. The non-traumatized uteri of rats on 1.5 mg. progesterone and 0.1 μg. estradiol weighed 122 mg., but when 1.0 μg. of the augmentor was added to the treatment the weight was increased to 195 mg. (Table 1). When the dosage of augmentor was increased to 5.0 and

10.0 μg. the average weights of the uteri were 176 and 177 mg., respectively. These weights are less than that attained when 1.0 μg. of augmentor was given, but overlapping of the standard errors indicates that these differences are not significant. If so, then it can be concluded that maximal potentiation of 0.1 μg. of estradiol occurred when 1.0 μg. of augmentor was given.

The data also show that the augmentor had a similar potentiating effect on the action of the other estrogens with which it was given. In each instance, the increase in weight of the non-traumatized horn showed a tendency to reach a plateau of response as the dosage of augmentor was increased, thus suggesting a possible quantitative relationship between estrogen dosage and amount of augmentor required to produce maximal potentiation. Our data are inadequate for an analysis of this possibility as only one dosage level for each estrogen was used in our experiments, but the general potentiating action of the augmentor raises an equally interesting question with respect to its effect on the interaction of estrogen and progesterone in the development of deciduomata. In our discussion of this it was mentioned that the further decrease in weight of deciduomata observed when the augmentor is added to an estrogen-progesterone treatment was probably due to a potentiation of estrogen thus enhancing its inhibitor influence on progesterone. This may be true for those estrogens that produce this reaction, but such an assumption does not explain the failure of the augmentor to influence the decidual response when estradiol benzoate and diethylstilbestrol were given even though it did potentiate the action of these estrogens as shown by an increase in weight of the non-traumatized uterine horn.

An additional feature with respect to the action of the augmentor is brought out when the data are compared on the basis of per cent increase of the traumatized over the non-traumatized horn (Table 1). It is obvious that so far as the inhibition of progesterone is concerned such treatment of the data gives a false impression of experimental fact. It is shown, for instance, that in castrated control rats given no hormone treatment, the traumatized horn of the uterus, as a response to injury, is larger than the non-traumatized horn. Also, it can be seen that in those groups given the augmentor plus estrogen and progesterone the increase in weight of the non-traumatized horn minimizes the per cent decrease of the decidual response and in this way exaggerates the inhibition, so much so, that when the data are compared in this fashion inhibition of decidual development is indicated although actual weights of deciduomata show definitely that this cannot be true. However, such consideration of the data does emphasize the ability of the augmentor to increase the effectiveness of estrogen in promoting uterine growth.

DISCUSSION

Our experimental results show that the estrogen-potentiating substance (augmentor) obtained by Cohen and Bates (1952) from the urine of pregnant mares, has the ability of intensifying the inhibitory action of certain estrogens on progesterone in the development of deciduomata in rats. A peculiar property of this substance, as reported by Cohen and Bates, is that it is non-estrogenic, in that it does not cause cornification of the vagina, but at higher dosages does stimulate growth of the uterus. The highest daily dose employed in our experiments was 54 μg. and this amount when given alone for three days did not produce a significant increase in weight of the non-traumatized uterine horn of castrated, pseudopregnant rats (Table 1). However, these are not the conditions under which Cohen and Bates tested their material, and our experiments were devised for another purpose.

We (Velardo and Hisaw, 1951) have shown that the ability of estrogens to inhibit progesterone in the development of deciduomata in rats is directly proportional to their estrogenic potency. So sensitive is this interaction that with graded doses of estradiol differences of 0.005 μg. can be detected. Therefore it seems a substance that increases the estrogenic action of an estrogen should increase at the same time its capacity to inhibit progesterone. The influence of the augmentor on this hormone interaction was tested by administering graded doses by separate injection in combination with an amount of estrogen that would reduce the decidual response to a daily dose of 1.5 mg. progesterone by about one half.

It was found that the augmentor in doses of 54 μg. does not modify the action of progesterone in the absence of estrogen nor, under these conditions, is there an increase in weight of the non-traumatized horn over that of non-treated, castrated controls. However, when a dosage as small as 1.0 μg. of the augmentor is given in combination with 0.1 μg. estradiol and 1.5 mg. progesterone there is a significant reduction of the decidual reaction beyond that produced by the estrogen alone. This ability to potentiate an estrogen is also demonstrated by a marked increase in weight of the non-traumatized horn of the uterus. The data in Table 1 show that these effects of the augmentor were obtained when it was given with estradiol, estrone, and estriol, but not when injected in combination with estradiol benzoate and diethylstilbestrol. However, it will be observed that although the augmentor did not increase the inhibitory action of either estradiol benzoate or diethlystilbestrol on decidual development it did increase the action of both of these estrogens on the non-traumatized horn. Therefore, it would appear that the increased inhibition of decidual development that occurs when the augmentor is administered concurrently with the three natural estrogens and progesterone may not be due entirely

to potentiation of estrogenic potency. Nor does the assumption that the augmentor is a weak estrogen fit all the facts, for if true it seems evident that its ability to potentiate known estrogens greatly exceeds its own estrogenic capacity.

SUMMARY

Cohen and Bates (1952) obtained a substance from the urine of pregnant mares which was non-estrogenic, in that it did not produce cornification of the vaginal mucosa of castrated rats, but did markedly augment the growth effects of estrogens on the uterus. It has been found that this substance is incapable of promoting a decidual response in the traumatized uterus of a castrated, pseudopregnant rat nor does it modify the decidual development induced by progesterone under these conditions. However, it does intensify the progesterone-inhibiting action of estradiol-17β, estrone, and estriol and bring about a corresponding, additional decrease in growth of deciduomata. In similar experiments, it does not potentiate the inhibitory action of estradiol benzoate or diethylstilbestrol on decidual growth even though the growth effects of these estrogens on the non-traumatized, contralateral uterine horn are markedly enhanced. The ability to increase the effectiveness of estrogens in promoting uterine growth may be a general property of the augmentor while its capacity to increase the inhibitory effects of estrogens on decidual development may apply only to certain compounds.

Acknowledgment

We are very grateful to Drs. Herman Cohen and Robert W. Bates for a generous supply of the Augmentor substance (lot No. 27-4-50).

REFERENCES

COHEN, H. AND R. W. BATES: *Endocrinology* **50**: 5. 1952.
COURRIER, R.: Vitamins and Hormones **8**: 179. 1950.
FRIEDEN, E. H. AND J. T. VELARDO.: *Proc. Soc. Exp. Biol. and Med.* **81**: 98. 1952.
HISAW, F. L. AND J. T. VELARDO.: *Endocrinology* **49**: 732. 1951.
VELARDO, J. T. AND F. L. HISAW. *Endocrinology* **49**: 530. 1951.

THE EFFECTS OF SEX HORMONES ON THE DEVELOPMENT OF THE RIGHT GONAD IN FEMALE FOWL[1]

ELSIE TABER AND KATHRYN W. SALLEY

Department of Anatomy, The Medical College of South Carolina, Charleston, South Carolina

INTRODUCTION

BIRDS, unlike other vertebrates, normally have but one functional ovary, located dorsally on the left side of the abdomen. In the domestic fowl, the right and left embryonic gonads develop concurrently until the 7–9th day of incubation at which time there is a retardation of growth, followed by retrogression, of the right organ (Swift, 1915; Brode, 1928). This right rudimentary gonad persists in the adult hen as an inconspicuous, flattened ridge of tissue, closely applied to the inferior vena cava. It is reported to be composed of medullary tissue, with cortical tissue rarely present (Willier, Gallagher and Koch, 1935; Domm, 1939; Snedecor, 1949). According to Lillie (1927), cortex is found only if regression of the right gonad is delayed until after the time proliferation of cortical cords normally occurs in the left ovary (8–11 days of incubation). Occasionally, this organ develops into a more or less typical right ovary. Domm (1939) reported that such ovaries, ranging in size from normal to barely visible, were found in less than 5% of adult brown Leghorn hens.

The exact factors involved in the embryonic inhibition of the right gonad are not understood, but it is probable that the left ovary exerts a restraining influence. The best evidence for this hypothesis comes from the effects produced by removal of the left ovary in birds after hatching. Although previous investigators had reported the results of ovariectomy, Domm (1927) was the first to make a careful analysis of the effects on the right gonad. It is now well established that, in the absence of the left ovary, the rudimentary right gonad develops into a testis-like organ, containing a variety of tubules, with or without lumina, and secreting androgen in quantities sufficient to cause masculinization of the head furnishings (comb and wattles) and crowing. Later in development, in addition to androgen,

Received for publication August 28, 1953.

[1] This investigation was supported by research grants from the Committee for Research in Problems of Sex, National Research Council, and by grant RG 2829 from the National Institutes of Health, Public Health Service.

the organ produces estrogen in quantities sufficient to bring about complete feminization of the plumage. Although this hypertrophied right organ may be called an "ovotestis", since it secretes large amounts of both androgen and estrogen, its histological structure almost always resembles that of a sterile or immature testis. Therefore, the term "ovotestis", as used in this paper to designate the typical right gonad found in sinistrally ovariectomized birds, refers to its endocrine function, rather than to its histological structure.

From this it seems clear that the ovary exerts an inhibitory influence on the development of the right gonad after hatching. There are several hypotheses to account for the nature of this influence and the purpose of the experiments reported here was to test one of these hypotheses: hormones (estrogen and/or androgen) produced by the ovary inhibit the development of the right rudimentary gonad.

Our results show that androgen or estrogen-therapy will either completely inhibit the development of the right gonad or will cause the organ to differentiate into a typical ovary or ovarian-like gonad.

EXPERIMENTS AND RESULTS

1. *Effects of estrogen*

a. Ovariectomy at 10–11 days of age. Ten single-comb, light-brown Leghorn chicks were ovariectomized on the 10 or 11th day after hatching, at which time a pellet of a-estradiol benzoate, weighing 25 mg., was implanted subcutaneously.[2] The new plumage, checked every 14 days, was of the female type throughout the course of the experiment, unless the pellets were purposely removed. Compared with ovariectomized or normal controls, the combs were small and pale, indicating suppression of androgen secretion. Exploratory operations performed on 6 birds between the 88th and 102nd day revealed no recognizable right gonad.

Laparotomies done on two birds on the 121st day disclosed a small (2.0×0.5 cm.), apparently normal ovary in one, and a small mass (0.2×0.1 cm.) of ovarian-like tissue in the other. The pellets were removed from these two birds at this time. Twenty-three days later the combs of both were larger and more vascular, indicating androgen production; the newly-formed feathers were of the neuter type, indicating a decreased level of estrogen. One bird was killed at this time. Grossly and histologically, the right gonad was an almost typical ovary, with a well-developed germinal

[2] Grateful acknowledgement is made to Dr. Norman L. Heminway of the Schering Corporation for the pellets of testosterone-propionate (Oreton-F) and estradiol benzoate (Progynon-B), and to Dr. M. H. Kuizenga of the Upjohn Company for the pregnant mare serum powder used in these experiments.

epithelium and containing many medium-sized follicles. There were, however, fewer large and small follicles than usually are found in the ovary of a normal pullet of the same age. This type right gonad will be referred to as "Type A ovary".

The other bird was killed at 174 days, at which time the comb was large and red, and the new plumage was feminine, indicating androgen and estrogen production. Grossly, the right gonad was a round, lobular organ, smaller than the "ovotestis" from an ovariectomized control of similar age. Several vesicles, filled with a straw-colored fluid, protruded from the surface. Histologically, the tissue resembled a semi-sterile ovary. Below a distinct germinal epithelium were many discrete nests of cells which might be called sterile follicles since the cells resembled normal follicular cells in every respect. The bulk of the gonad was composed of closely-packed cords of cells in which were embedded several well-developed follicles containing normal-appearing ova. A few medullary tubules were observed in one lobe, but, in general, the stroma was ovarian in nature. This type right gonad will be referred to as "Type B semi-sterile ovary".

In the remaining 8 birds, killed at 144 days, no gonadal tissue was found.

b. Ovariectomy at 21–22 days of age. Ten chicks were ovariectomized and inplanted with a pellet of a-estradiol benzoate (25 mg.) on the 21st or 22nd day. Compared with ovariectomized controls, their combs were small and pale; new plumage was of the female type.

Between 70 and 92 days, exploratory operations revealed 5 birds with no gonadal tissue, one with a definite right ovary (0.6×0.2 cm.), three with small masses (averaging 0.3×0.2 cm.) which appeared to be ovarian, and one with gonadal tissue too small to be identified grossly as ovarian (see Table 1). There was no correlation between the age at the time of operation and the amount or kind of gonadal tissue present. Similar operations on 4 ovariectomized controls disclosed typical "ovotestes".

At 103 days, the pellets were removed from two of the 5 birds observed to have gonadal tissue at the time of the laparotomies. Similarly, pellets were removed from three of the 5 birds which had shown no gonadal tissue. There was an increase in the rate of comb growth in both groups and, within 13 days, the new feathers were of the neuter type. Of the two birds known to possess gonadal tissue, the new plumage again became feminine 4 or 6 weeks after the pellets were removed, indicating endogenous estrogen. One of the three which had no gonadal tissue at the time of laparotomy had plumage of the female-type 8 weeks after the pellet was removed.

All of the birds were killed at 161 days. Each bird was examined grossly for gonadal tissue, which, if present, was sectioned and studied histologically. The 7 ovariectomized controls had female plumage, large, red combs

TABLE 1. EFFECTS OF ANDROGEN AND ESTROGEN ON OVARIECTOMIZED FOWL

Bird	Rt. gonad at 70–92 days	Rt. gonad at autopsy 161 days		Comb height × length	New plumage	Pituitary ant. lobe
		Ovariectomized Controls				
20	ovotestis	ovotestis	296.2 mg.	59.2 cm.	Fem.	9.0 mg.
22	ovotestis	ovotestis	185.6	55.4	Fem.	13.1
23	(not examined)	ovotestis	313.6	73.5	Fem.	12.9
24	(not examined)	ovotestis	352.0	66.0	Fem.	10.9
25	ovotestis	ovotestis	249.7	48.5	Fem.	13.6
26	ovotestis	ovotestis	—	—	Fem.	—
27	(not examined)	ovotestis	244.2	29.4	Fem.	13.1
Avg.			273.6	55.3		12.0
		Ovariectomized + Estrogen Pellet				
18	small?	"A" ovary	3.0	5.7	Fem.	4.1
10	small-ovarian	"A" ovary	5.3	4.8	Fem.	5.9
16	medium-ovarian	"A" ovary	76.4	5.6	Fem.	6.3
12	0	0	—	4.7	Fem.	7.3
14	0	0	—	6.7	Fem.	7.4
Avg.				5.5		6.2
		Pellets Removed at 103 Days				
11	small-ovarian	"B" ovary	35.0	6.1	Fem.	7.8
13	small-ovarian	"A" ovary	184.0	12.3	Fem.	7.6
15	0	0	—	8.0	Neuter	11.0
17	0	0	—	11.5	Neuter	13.4
19	0	ovotestis	168.2	15.1	Fem.	11.6
Avg.				10.6		10.3
		Ovariectomized + Androgen Pellets				
9	small-ovarian	"A" ovary	11.9	106.1	Neuter	7.6
5	small-ovarian	"A" ovary	40.9	88.2	Fem.	8.4
0	0	0	—	78.4	Neuter	5.5
4	0	0	—	88.4	Neuter	7.7
6	0	0	—	58.7	Neuter	8.5
Avg.				84.0		7.5
		Pellets Removed at 103 Days				
3	small-ovarian	"B" ovary	62.4	27.7	Fem.	11.1
8	small ?	"B" ovary	115.3	19.5	Fem.	7.8
7	?	ovotestis	57.0	24.9	Neuter	13.9
2	?	ovotestis	63.9	35.9	Fem.	13.2
1	0	ovotestis	90.6	31.5	Neuter	15.9
Avg.				27.9		12.4

(average height × weight—55.3 cm.^{2}), and typical "ovotestes" averaging 273.6 mg. in weight (see Table 1). These observations agree with those made previously in this laboratory on a series of 50 ovariectomized birds.

Of the 5 experimental birds known to contain gonadal tissue, the comb-

size of the three in which the pellets remained averaged 5.4; the average comb-size of the two from which the pellets were removed was 9.2. This suggests that the pituitaries of the latter birds had recovered from the inhibitory effects of the high estrogen levels and were stimulating the gonadal tissue to secrete androgen. The right gonads from the three under continuous estrogen stimulation weighed 3.0, 5.3 and 76.4 mg., and could be described as "Type A ovaries". In the two from which the pellets were removed 58 days prior to autopsy, the gonads weighed 35.0 and 184.0 mg., the former being a "Type B semi-sterile ovary" and the latter a "Type A ovary".

The remaining 5 birds had no gonadal tissue at the time of laparotomy. Two of these, under continuous estrogen stimulation, had combs averaging 5.7 and again were found to be free from gonadal tissue. Of the other three birds, from which the pellets had been removed, two showed no gonadal tissue but had combs measuring 9.8. This suggested the possibility that adrenal androgen might be responsible for the increased comb-size. The third one, with a red, erect comb (15.1) and female plumage, had developed a typical "ovotestis" weighing 168.2 mg.

2. *Effects of androgen*

Ten birds were ovariectomized and implanted with pellets of testosterone propionate, weighing approximately 38 mg., on the 21st or 22nd day. Within a few days the combs became red and turgid and rapidly increased in size. A second pellet was implanted on the 74th or 88th day, when a slight decrease in the comb-size indicated a decrease in the degree of androgen-stimulation. The new plumage of all birds was of the neuter-type.

Laparotomies performed between the 70th and 92nd day disclosed the following: 4 birds with no gonadal tissue, three with small ovarian-like gonads (0.3×0.1 cm.), two with hypertrophied Wolffian bodies and possibly a small mass of gonadal tissue, and one with gonadal tissue too small to be identified grossly.

Pellets were removed from 5 birds on the 103rd day. This was followed by a decrease in the size, turgidity and vascularity of the combs. All birds were killed on the 161st day. Hypertrophied right gonads were found in each of the 5 birds from which the pellets had been removed. Three of these, believed to be free of gonadal tissue at the time of laparotomy, had developed small, typical "ovotestes" weighing 63.9, 90.6, and 57.0 mg. The first of these was secreting estrogen in quantities sufficient to cause feminization of the plumage by the 158th day. The two birds with small masses of gonadal tissue at the time of laparotomy had developed "Type B semi-sterile ovaries" which weighed 62.4 and 115.3 mg., and

which had been producing estrogen (indicated by female plumage) since the 130th day.

Of the 5 birds under continuous androgen stimulation (comb size 84.0), the three birds which had been free from gonadal tissue at the time of laparotomies remained free. The other two had developed "Type A ovaries" weighing 11.9 and 40.9 mg. Since the larger ovary was producing sufficient estrogen to cause feminization of the new feathers, the inhibition of the pituitary, caused by the high androgen level, could not have been complete.

3. *Effect of estrogen and androgen on the pituitary*

The average weights of the anterior lobes of the pituitaries from birds under continuous estrogen or androgen stimulation were 6.2 and 7.5 mg., respectively. The average weights of these glands in birds from which the estrogen or androgen pellets were removed 58 days prior to autopsy were 10.3 and 12.4 mg., respectively. The anterior lobes of ovariectomized controls averaged 12.0 mg. Although the glands have not been studied histologically, the decrease in weight of the organs in the hormone-treated birds suggests pituitary inhibition.

One preliminary experiment, in which two androgen-treated ovariectomized birds, two estrogen-treated ovariectomized birds, and two ovariectomized controls were injected with equine gonadotrophin, revealed no difference between the condition of the gonads in treated birds and in similar, untreated controls. Since the fowl is known to become refractory to equine gonadotrophin when it is administered over a long period of time (Taber, 1948), these results are not considered significant, and more precise experiments along these lines are in progress.

DISCUSSION

The rudimentary right gonad in the sinistrally ovariectomized birds treated with estrogen or androgen in these experiments failed to develop in 65% and 60% of the cases, respectively, or developed into an ovarian-like organ, containing normal-appearing follicles. All ovariectomized controls developed typical, sterile "ovotestes".

In a recent abstract, Kornfeld and Nalbandov (1953) reported that estrogen-treatment completely or partially suppressed the development of the right gonads in ovariectomized white Leghorns; androgen treatment likewise suppressed development, but to a lesser degree. No description of the histological structure of the gonads was given.

The high percentage of ovaries in our experiments would not be expected by chance. Domm (1937) reported only three cases of ovaries developing at the site of the rudimentary gonad in "several hundred" ovariectomized

birds, and Gray (1930) observed no follicles in 87 right gonads, 36 of which were serially sectioned. In our laboratory, we have seen 7 right ovaries in 163 ovariectomized birds—approximately 4%.

The complete suppression of the right gonad in 62% of our treated birds and the small size of the gonads in the remaining birds suggested a decrease in the amount of available gonadotrophins. The pronounced reduction of pituitary weight also indicated inhibition of pituitary function. After the pellets were removed from 6 birds, in which exploratory operations had revealed no hypertrophied right gonad, 4 developed typical "ovotestes" within 58 days, and pituitary weights were normal.

The fact that ovarian tissue, rather than the cords and tubules of an "ovotestis", developed in 38% of the treated birds is not easy to explain on the basis of pituitary inhibition alone. The hypothesis that the cortical component of the gonad has a lower threshold for response to gonadotrophic stimulation than does the medullary component does not agree with other observations (Taber, 1948).

Since the right rudimentary gonad in the young chick resembles an embryonic gonad in some respects, one might expect that treatment with sex hormones would modify the rudimentary gonad and the embryonic gonad in a similar manner. Therefore, the presence of ovarian-like gonads in estrogen-treated ovariectomized birds is not surprising, when one considers that estrogen transforms the left testis of the embryo into an organ almost indistinguishable from a normal ovary. The right testis, however, never completely loses its testicular nature (Kozelka and Gallagher, 1934; Willier, Gallagher, and Koch, 1935, 1937; Snedecor, 1949). Although most investigators agree that estrogen has no effect upon the embryonic ovary, a well-defined cortex on the right gonad has been observed in a few cases (Willier, *et al.*, 1935, 1937; Dantchakoff, 1936; Snedecor, 1949).

There is some disagreement as to the effects of testosterone. Willier, Rawls and Koch (1938) observed that testosterone resulted in degeneration of the cortex and hypertrophy of the medulla of the embryonic ovary but had little effect upon the testis. Wolff and Wolff (1937) reported that high concentrations of testosterone produced masculinizing effects on the ovary, but produced feminizing effects on the testis of Leghorns, although certain other varieties of fowl were unaffected. It is agreed that androsterone and dehydroandrosterone exert a strong feminizing influence on the testis and a masculinizing effect on the left ovary (Willier *et al.*, 1935, 1937, 1938; Wolff, 1935, 1936; Wolff and Wolff, 1937). Occasionally, an increased amount of cortical tissue was observed on the right gonad (Willier *et al.*, 1935, 1937).

It has been generally accepted that "sex hormones do not originate a morphological component but merely activate one already laid down in

normal development" (Willier *et al.*, 1935). If this statement is correct, treatment with androgen or estrogen in these experiments directly or indirectly "activated" cortical tissue already present in the rudimentary gonad. The cortical tissue may have been stimulated primarily and it, secondarily, inhibited the differentiation of the medullary component; or the treatment may have inhibited the development of the medullary portion, which, secondarily, "permitted" the cortical element to grow and differentiate. The latter hypothesis seems more likely, since, in our experiments, the development of medullary tissue was inhibited whether or not cortical tissue was shown to be present.

This leads to the conclusion that cortical tissue was present in the rudimentary gonads of 38% of the treated birds. This is not in accord with the generally accepted belief that the right gonad in the female "is usually devoid of cortex" (Lillie, 1927), or "normally lacks ovarian cortex" (Snedecor, 1949), or that cortical tissue is "typically quite rudimentary or absent" (Willier *et al.*, 1935) or is found only in "exceptional" (Domm, 1939) or "rare cases" (Brode, 1929). In the same paper Brode stated: "In 39% of the specimens cortical rudiments were present". This statement appears contradictory to his other conclusion, but is in close agreement with the results of our experiments.

The possibility that a rudimentary cortex was not originally present, but was induced by the hormone treatment can not be completely ignored. Lewis (1946) postulated that cortex could be formed from primary testis cords in estrogen-treated duck embryos. If this is true in the fowl, it is possible that the androgen and estrogen treatment resulted in the conversion of medullary tissue into cortical.

In our experiments, another interesting observation was the presence of a large number of normal-appearing germ cells in the ovarian gonads of the treated birds, while all of the control "ovotestes" were sterile.

Swift (1915, 1916) demonstrated that the definitive sex cells arose from primordial germ cells, and Brode (1928) described the presence of such cells in abundance in both the cortex and the medulla of right gonads from chicks up to three weeks of age. Since they gradually disappeared in older birds, Domm (1927, 1929) attributed the sterility of most "ovotestes" to the absence of primordial germ cells in the rudimentary organ at the time the left ovary was removed.

The chicks in our experiments were ovariectomized during the first three weeks, at which time primordial germ cells should have been present in the rudimentary gonads. The fact that oocytes were seen in all of the ovarian gonads but no germ cells were observed in the controls suggested that cortical tissue might provide a more favorable environment for the survival and development of the primordial germ cells.

SUMMARY

Thirty-seven brown Leghorn chicks were ovariectomized during the first three weeks after hatching. Twenty of these were implanted with pellets of estradiol benzoate, and 10 with pellets of testosterone propionate at the time the ovary was removed.

The rudimentary right gonad of each of the seven controls developed into a typical "ovotestis" composed of sterile testis-like tubules and cords of cells, and secreting androgen and estrogen in quantities sufficient to produce large, erect, red combs and feminine plumage, respectively.

The development of the rudimentary gonad was inhibited in 65% of the estrogen-treated and 60% of androgen-treated birds. Thirty-five per cent of the estrogen-treated, and 40% of the androgen-treated developed ovarian-like gonads, containing follicles. When the pellets were removed from birds in which no gonadal tissue was noted at 92 days, 67% developed typical "ovotestes". There was a significant reduction in the weight of the anterior lobe of the pituitaries from treated birds.

It was concluded that the sex hormones, acting directly or indirectly on the gonadal tissue, inhibited the medullary component (whether or not cortex was shown to be present), and either stimulated a pre-existing cortical component, or caused the formation and subsequent development of new cortical tissue. It is possible that the mechanism responsible for these effects was similar to that by which these hormones modify the development of embryonic gonads.

If these hormones did not initiate the formation of new cortical tissue it must be concluded that it was already present in 38% of the rudimentary gonads of the hormone-treated birds. Therefore, it seems possible that the presence of a rudimentary cortex is not an exceptional condition, as is commonly stated.

REFERENCES

BRODE, M.D.: *J. Morphol. and Physiol.* **46:** 1. 1928.
DANTCHAKOFF, V.: *Compt. rend. Soc. de biol.* **122:** 168. 1936.
DOMM, L. V.: *J. Exper. Zool.* **48:** 31. 1927.
DOMM, L. V.: *Arch. f. Entwcklngsmechn. d. Organ.* **119:** 171. 1929.
DOMM, L. V.: Sex and Internal Secretions (E. Allen, ed.). Chap. 5: 227. 1939.
GRAY, J. C.: *Am. J. Anat.* **46:** 217. 1930.
KORNFELD, W. AND A. V. NALBANDOV: *Anat. Rec.* **115:** 337. 1953.
KOZELKA, A. W. AND T. F. GALLAGHER: *Proc. Soc. Exper. Biol. and Med.* **31:** 1143. 1934.
LEWIS, L. B.: *Physiol. Zoöl.* **19:** 282. 1946.
LILLIE, F. R.: *J. Exper. Zool.* **48:** 175. 1927.
SNEDECOR, J. G.: *J. Exper. Zool.* **110:** 205. 1949.
SWIFT, C. H.: *Am. J. Anat.* **18:** 441. 1915.
SWIFT, C. H.: *Am. J. Anat.* **20:** 375. 1916.
TABER, E.: *J. Exper. Zool.* **107:** 65. 1948.

WILLIER, B. H., T. F. GALLAGHER AND F. C. KOCH: *Proc. Nat. Acad. Sc.* **21**: 625. 1935.
WILLIER, B. H., T. F. GALLAGHER AND F. C. KOCH: *Physiol. Zoöl.* **10**: 101. 1937.
WILLIER, B. H., M. E. RAWLES AND F. C. KOCH: *Proc. Nat. Acad. Sc.* **24**: 176. 1938.
WOLFF, E.: *Compt. rend. Acad. d. sc.* **201**: 1055. 1935.
WOLFF E.: *Compt. rend. Soc. de biol.* **121**: 1474. 1936.
WOLFF, E. AND E. WOLFF: *Compt. rend. Soc. de biol.* **124**: 367. 1937.

ROLE OF HYPOGLYCEMIA AND CARBON DIOXIDE IN THE REACTION TO STRESS*

L. L. LANGLEY, P. W. SCOKEL AND E. J. MOORE

Department of Physiology, Medical College of Alabama and the University of Alabama School of Dentistry, Birmingham, Alabama

STRESS evokes increased activity of the adrenal cortices. The sequence of events by which this end result is brought about has yet to be outlined. Under the stress of hypoxia it has been shown that the observed alterations are not due to the hypoxemia *per se*, but rather to the resulting hypocapnia (Langley *et al.*, 1950). Long (1947) suggested that a common element in the various forms of stress may be the increased output of the adrenal medulla. Some time ago Lewis *et al.* (1942) presented evidence which indicated that hypoxia caused hypoglycemia and the hypoglycemia triggered the pituitary-adrenal axis. Recent experiments implicate the hypothalamus in this sequence of events (Porter, 1953). But despite the assault on this problem spreading over two decades, the mechanism remains obscure. For this reason a series of experiments were carried out in an attempt to shed some light upon the role of hypoglycemia and carbon dioxide in the reaction to stress.

METHODS

Two types of stress were used: (1) hypoxia and (2) cold. Hypoxia was produced in adequately ventilated decompression chambers at a simulated altitude of 20,000. The cold experiments were carried out in a large, thermostatically controlled room set at 3° C. Male rats (150–200 grams) of the Sprague-Dawley strain were used for the hypoxia studies. Male rats of the same weight of the Long-Evans strain were used in the cold experiments.

For the glucose studies blood was obtained by cutting the tail of unanesthetized rats and analyzed by the Folin (1929) modification of the Folin-Wu method. In the liver glycogen series the animals were given a full anesthetic dose of sodium pentothal and about one gram of liver removed for analysis by the method of Cori (1932). Ascorbic acid was determined by the Roe and Kuether procedure (1943), cholesterol (Sperry, 1938), and eosinophils (Randolph, 1944).

RESULTS

Blood Sugar: All animals were fasted 24 hours before exposure to low oxygen tension. Blood sugar was then determined after exposures of 1, 2, 4,

Received for publication September 1, 1953.

* These results were presented before the International Physiology Congress in Montreal, 1953.

and 24 hours. The results are illustrated in Figure 1. It can be seen that there is a transient fall in blood sugar, but by the end of 4 hours it has returned to normal, while after 24 hours there is a slight hyperglycemia.

Carbon Dioxide: We have previously reported that carbon dioxide prevents the liver glycogen response to high altitude (Langley *et al.*, 1950). Hailman (1944) demonstrated that prevention of hypocapnia blocks the expected hypertrophy of the adrenal glands in rats exposed to hypoxia. The question arose as to whether this finding had any significance under other stress conditions. On the assumption that severe stress of any kind produces hyperventilation and therefore hypocapnia, cold, instead of hypoxia was used as a stressing agent. Adrenal activity was followed by determining adrenal ascorbid acid, cholesterol, and the number of eosinophils. Three groups of rats were analyzed: (1) controls fasted 4 hours at room temperature, (2) those fasted 4 hours in the cold room, and (3) those fasted four hours in the cold room in which the atmosphere contained 5% CO_2. All animals were fasted 8 hours before beginning the runs. The results are tabulated in Figure 2. It can be seen that cold exposure brings about a sharp drop in ascorbic acid, cholesterol, and the number of eosinophils. When CO_2 is added to the cold atmosphere the drop proves to be significantly less, but they are still significantly lower than the control

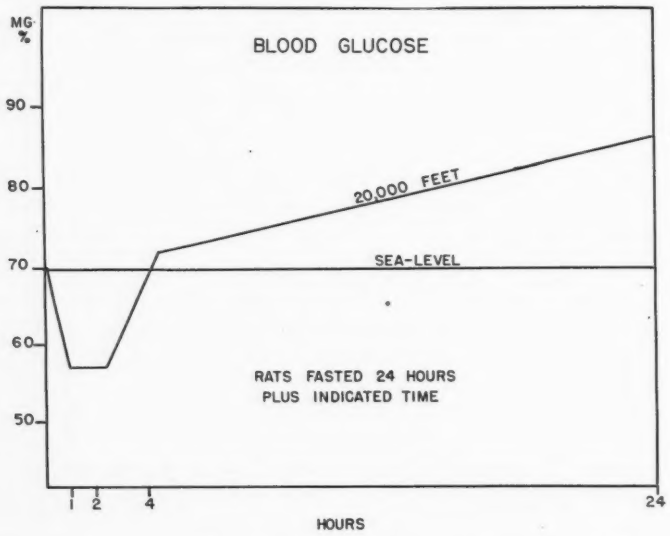


FIG. 1. Transient hypoglycemia due to hypoxia. All animals were fasted 24 hours before being placed in the chambers and exposed to a simulated altitude of 20,000 feet.

EXPERIMENTAL CONDITIONS	ASCORBIC ACID (mg/100 gm)	CHOLESTEROL (mg/100 mg)	EOSINOPHILS
CONTROL (ROOM TEMP)	394 ± 7.5	1766 ± 220	63 ± 3.2
COLD (2–4° C)	293 ± 12.3	1138 ± 80	6 ± 1.2
COLD + 5% CO_2	328 ± 9.5	1600 ± 80	34 ± 5.8

FIG. 2. The adrenocortical response to cold, and cold plus an atmosphere containing 5 per cent carbon dioxide.

values. This must be interpreted to mean that carbon dioxide prevents, but not completely, the adrenal activation due to cold.

DISCUSSION

The above results suggest that in the reaction to stress there is (1) transient hypoglycemia, and (2) that alterations in the blood carbon dioxide content are of significance. Previously (1942) we reported that in response to the stress of hypoxia the urinary excretion of sodium, potassium, and water is markedly increased. Constantinides (1951) has demonstrated renal hypertrophy in response to stress; hypertrophy which does not occur in the adrenalectomized and untreated animal. Roemmelt *et al.* (1949) showed that adrenalectomized rats do not excrete a large sodium load as well as normal animals do. Figure 3 shows results previously reported (Langley *et al.*, 1952). It can be readily seen that nephrectomy prevents the liver glycogen accumulation in response to hypoxia. Porter (1953) reports that lesions placed in the posterior part of the hypothalamus blocks the adrenal reaction to stress. He also demonstrated typical eosino-

penia when this area was electrically stimulated. On the other hand Cheng *et al.* (1949) find that pituitary stalk section or pituitary transplant does not interfere with the adrenocortical response to stress. Finally Fortier (1951) reports that a transplanted anterior pituitary responds normally to stress such as cold, adrenalin, and histamine, but not to sound or immobilization.

When one contemplates these diverse observations he is tempted to conclude that as yet two basic questions cannot be answered. They are: (1) what is the sequence of events by which stress evokes adrenocortical

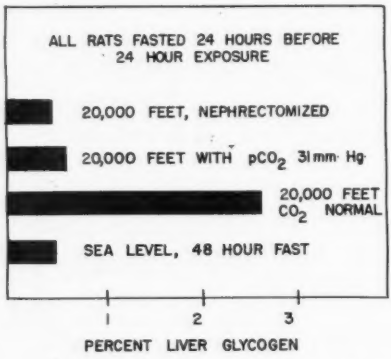


FIG. 3. Liver glycogen response to hypoxia. Carbon dioxide and nephrectomy are seen to prevent the normal accumulation.

activation, and (2) how does the resulting outpouring of adrenocortical hormones assist the animal in adapting to these adverse conditions? True these questions cannot be answered with finality and certainty. But in many clues which have accumulated during the past two decades several common denominators suggest possible answers (Fig. 4).

In both the high altitude chamber and the cold room it is easy to observe hyperventilation. However, actual ventilation should be ascertained. Even more important would be blood studies under varying stress conditions to determine if significant hypocapnia does, in fact, develop. In Figure 4 there are dual and triple mechanisms portrayed. It is highly conceivable that certain stress conditions, such as immobilization, or loud noises, may result in a barrage of impulses being directed to the hypothalamus. As a result the adrenal medulla could be stimulated, or neurohumors could directly stimulate the anterior pituitary. We are forced to the conclusion that the response to stress involves several lines of defense, as do so mnay other physiological mechanisms.

The weight of experimental data strongly suggests that the output of

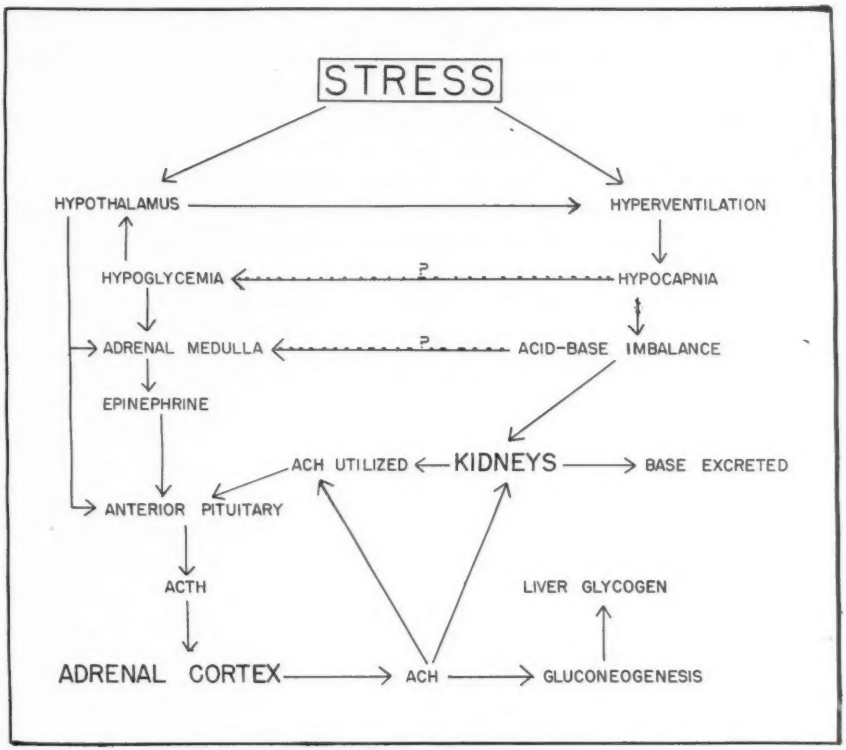


FIG. 4. A suggested sequence of events by which stress activates the adrenal cortex.

ACTH is controlled by (1) epinephrine, (2) ACH, and (3) neurohumors from the hypothalamus. Thus in some stress conditions there may simply be hypoglycemia. This alone could activate the adrenal medulla thus producing epinephrine. If the stress is mild and if there is adequate glycogen in the liver, the blood sugar will be quickly returned to normal. On the other hand greater stress under conditions of liver glycogen depletion may evoke gluconeogenesis, a process which probably utilizes ACH. The circulating epinephrine plus a diminished blood ACH would prove effective in activating the output of ACTH. Finally, in severe stress the hypothalamus would be stimulated by direct neural pathways and, in addition, the resulting hyperventilation would bring about acid-base imbalance with appropriate renal compensation. This compensation also probably utilizes ACH resulting in diminished circulating ACH and consequent anterior pituitary activation.

SUMMARY

The role of hypoglycemia and carbon dioxide in the reaction to hypoxia and cold was investigated in male rats. It was found that hypoxia produces a transient hypoglycemia which reaches a maximum after 4 hours exposure to 20,000 feet. Thereafter the blood sugar returns to normal.

Rats exposed to 3° C. for 4 hours showed depletion of adrenal ascorbic acid, cholesterol, and eosinopenia. These responses can be partially blocked if the concentration of carbon dioxide in the atmosphere is kept at 5%.

These results combined with previous work and other published data are discussed and the suggestion offered that there are at least three lines of defense open to the stressed animal. It is believed that (1) adrenaline is secreted, (2) ACH is utilized and thus decreased in the circulation blood, and (3) the hypothalamus is stimulated directly. In severe stress hypocapnia probably ensues which potentiates these mechanisms.

REFERENCES

CHENG, C., G. SAYERS, L. S. GOODMAN AND C. A. SWINYARD: *Am. J. Physiol.* **158:** 45. 1949.

CHENG, C., G. SAYERS, L. S. GOODMAN AND C. A. SWINYARD: *Am. J. Physiol.* **159:** 433. 1949.

CONSTANTINIDES, P.: *Endocrinology.* **49:** 512. 1951.

CORI, G. T.: *J. Biol. Chem.* **96:** 259. 1932.

FOLIN, O.: *J. Biol. Chem.* **82:** 83. 1929.

FORTIER, C.: *Endocrinology.* **49:** 782. 1951.

HAILMAN, H. F.: *Endocrinology.* **34:** 187. 1944.

INGLE, D. J.: *J. Endocrinology.* **8:** xxiii. 1952.

LANGLEY, L. L., L. G. NIMS AND R. W. CLARKE: *Am. J. Physiol.* **161:** 331. 1950.

LANGLEY, L. L., P. W. SCOKEL AND J. A. WHITESIDE: *Fed. Proc.* **11:** 88. 1952.

LANGLEY, L. L. AND R. W. CLARKE: *Yale J. Biol. & Med.* **14:** 529. 1942.

LEWIS, R. A., G. W. THORN, G. F. KOEPF AND S. S. DORRANCE: *J. Clin. Investigation.* **21:** 33. 1942.

LONG, C. N. H.: *Bull. New York Acad. Med.* **23:** 260. 1947.

PORTER, R. W.: *Am. J. Physiol.* **172:** 515. 1953.

RANDOLPH, T. G.: *J. Allergy.* **15:** 89. 1944.

ROE, J. H. AND C. A. KUETHER: *J. Biol. Chem.* **147:** 399. 1943.

ROEMMELT, J. C., O. W. SARTORIUS AND R. F. PITTS: *Am. J. Physiol.* **159:** 124. 1949.

SPERRY, W. M.: *Am. J. Clin. Path.*, Tech. Suppl. **2:** 91. 1938.

A STUDY OF THE LYMPHOCYTOLYTIC ACTION OF AN ADRENAL CORTICAL EXTRACT *IN VITRO*[1]

ZELMA MILLER

With the technical assistance of NANCY McINTOSH

The Children's Cancer Research Foundation, the Children's Medical Center, and the Department of Pathology, Harvard Medical School, Boston, Massachusetts

A COMMERCIAL adrenal extract, Lipo-Adrenal Cortex in cottonseed oil, has been reported to possess marked lymphocytolytic activity *in vitro*. This property is not manifested by other similar preparations nor by any of several pure hormones studied (Schrek, 1949; Hechter and Johnson, 1949; Feldman, 1950).

The aerobic glycolysis of rat thymus lymphocytes is also stimulated by this extract at concentrations which are too low to influence either anaerobic glycolysis or respiration (Miller, 1953 and 1954). The increased aerobic glycolysis cannot be attributed to cortisone, hydrocortisone, or desoxycorticosterone (Miller, 1953 and 1954, and Hechter and Miller, unpublished). Inhibition of respiration was observed with higher concentrations of Lipo-Adrenal Cortex, as well as with cortisone and hydrocortisone.

These results suggest the possibility that lymphocytolysis and the stimulation of aerobic glycolysis might be related, and are perhaps referable to the same component or components of Lipo-Adrenal Cortex. A study has therefore been made of possible correlations between these two phenomena.

METHODS

Thymus lymphocytes for each experiment were obtained by mincing the thymus gland from a normal young Hisaw rat in cold Ringer-Krebs phosphate buffer (Krebs and Henseleit, 1933) in an ice-bath. The suspension was diluted to the desired volume, usually 15 to 20 cc., and filtered through several layers of gauze. In most experiments, cells were separated more completely from stroma by plunging the piston of a very loose-fitting Potter-Elvehjem glass homogenizer into the diluted mince several times before filtering.

Experiments were conducted in 20 cc. beakers in the Dubnoff Metabolic Shaking Incubator (Dubnoff, 1948), operated at low speed to avoid mechanical damage to the cells. The beakers generally contained 1 cc. cell suspension, sufficient glucose to provide a final concentration of M/50, 0.05 or 0.1 cc. of L.A.C.[2] or cottonseed oil, and Ringer-

Received for publication September 4, 1953.

[1] Aided by a grant from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

[2] For convenience, Lipo-Adrenal Cortex will be referred to as L.A.C.

Krebs phosphate buffer to 1.5 cc. The experimental period was one hour, and the temperature, 37° C. The vessels were gassed with oxygen.

Aerobic glycolysis, $Q_A^{O_2}$ (mm.^{3} CO_2 evolved, equivalent to lactic acid, per mg. dry weight per hour) was calculated from chemical estimation of lactic acid. Aliquots were withdrawn at the end of the incubation period for lactic acid determination and cell counts. After precipitation of proteins with CCl_3COOH, lactic acid was determined by the method of Miller and Muntz (1938), as modified by Barker and Summerson (1941). Dry weight of the lymphocyte suspension in Ringer-Krebs medium was estimated by drying a representative sample and correcting for the dry weight of the salts present.

Lymphocyte viability was determined according to Schrek (1949) with 1:4000 safranin. Cells which are stained by safranin are considered to be non-viable.

All experiments with Lipo-Adrenal Cortex in cottonseed oil were compared with controls, containing the same volume of the cottonseed oil vehicle. We are indebted to Dr. H. F. Hailman of the Upjohn Company for generous supplies of Lipo-Adrenal Cortex in cottonseed oil and in absolute alcohol, as well as for the cottonseed oil vehicle.

Safranin O was a product of the Allied Chemical and Dye Corporation, National Aniline Division, C.I. #841.

RESULTS

Determination of the percentage of stained cells

Most investigators who have studied the influence of adrenal cortical extracts and purified hormones on lymphocytolysis have expressed their results as the percentage of cells which have become permeable to and therefore stainable by eosin or safranin. These have been designated "non-viable" cells. The techniques used follow those of Schrek (1946a, 1946b, and 1949). Thymus lymphocytes, washed or unwashed, are incubated with the adrenal cortical extracts or hormones, usually in the presence of homologous serum or plasma. The percentage of stained cells is then determined by counting the total number of cells with cresyl blue, and the number of cells stained by eosin or safranin. Feldman (1950) noted that "cells freed of plasma and suspended in various physiological media are apparently somewhat injured, for they show a very faint yellow-orange hue when diluted with the eosin-containing fluid."

Our experiments on aerobic glycolysis by lymphocytes have all been conducted on unwashed cells, suspended in inorganic medium without added serum or plasma, because these constitute sources of substrate and preformed lactic acid which are undesirable for our purposes. In attempting to correlate increased glycolysis with the percentage of cells permeable to eosin or safranin, we also observed this rather generalized, non-specific staining, indicating increased permeability in a large number of cells.

Further study of this phenomenon revealed that the number of cells permeable to eosin or safranin[3] increases rapidly with time. Thus, in a typical

[3] Safranin was used in preference to eosin because counts were made more easily with this stain.

experiment, an ice-cold suspension of cells was mixed with 7 volumes of ice-cold 1:4000 safranin, and the cells were counted as rapidly as possible. Five and a half percent of the cells were "non-viable" by this technique. This value increased to 36% after 5 minutes, 77% after 10 minutes, and 97% after 15 minutes.

In confirmation of the work of other investigators, it was found that fewer cells were stained if the lymphocytes were washed and/or incubated in a mixture of 3 volumes Ringer-Krebs phosphate buffer plus 2 volumes homologous serum or plasma.

Our experiments indicated, however, that the serum or plasma need not be present from the beginning of the incubation period. If it is added just

TABLE 1. INFLUENCE OF PLASMA ON PERMEABILITY OF RAT LYMPHOCYTES TO SAFRANIN

Cells were suspended in Ringer-Krebs phosphate buffer, pH 7.4. Plasma was added just prior to safranin

Lymphocyte suspension, cc.	Safranin 1:4000, cc.	Rat plasma, cc.	Ringer-Krebs phosphate buffer, cc.	% cells stained minutes	
				0	5
0.5	3.5	0.0	0.1	23	90
0.5	3.5	0.1	0.1	11	18

prior to or simultaneously with the dye, it protects the cells equally well (Table 1).

It is not necessary to use homologous serum or plasma. Dog serum is as effective as rat serum or plasma. Serum and plasma cannot be replaced by purified lecithin,[4] by an ultrafiltrate of serum or by gelatin. However, crystalline bovine plasma albumin[4] is very effective. In a typical experiment with 1:4000 safranin which contained approximately 1 mg. bovine plasma albumin per cc., the initial count of a suspension of unwashed thymus lymphocytes in Ringer-Krebs phosphate buffer showed a low percentage of stained cells, 8%. This value did not increase significantly after 30 minutes. The routine procedure adopted, therefore, was to mix one volume of diluted lymphocyte suspension with 7 volumes of 1:4000 safranin which contained 1 mg. bovine plasma albumin/cc. The counting chamber was filled, and approximately 200 cells were counted for each determination. With carefully prepared cell suspensions, free of clumps, the maximum variation in total cell count for duplicate experiments amounted to 10%. The variation in the percentage of cells permeable to safranin was less than 5%.

[4] I am indebted to Dr. Lahut Uzman for samples of highly purified crystalline bovine plasma albumin and purified lecithin.

Correlations of cell permeability with aerobic glycolysis

Under the conditions of our experiments, which are conducted for only one hour, L.A.C. can produce marked changes in aerobic glycolysis with little or no increase in the number of cells permeable to safranin (Table 2, Expts. 1 and 2).

In order to rule out the possibility that the use of cottonseed oil as a vehicle influences either of these reactions, experiments have also been performed with the active material dissolved directly in the Ringer-Krebs medium. For this purpose, an alcoholic extract of L.A.C. is evaporated to dryness *in vacuo* at room temperature, and the residue is redissolved by

TABLE 2. CHANGES IN AEROBIC GLYCOLYSIS AND PERMEABILITY TO SAFRANIN AFTER EXPOSURE TO LIPO-ADRENAL CORTEX

Expt. No.	cc. added/1.5 cc.	Aerobic glycolysis $Q_A^{O_2}$		Cells stained by safranin %	
		Control	L.A.C.	Control	L.A.C.
1*	0.1	1.5	2.7	15	15
2*	0.2	1.8	3.3	4	13
3†	(0.05)	2.0	4.1	6	6

Ringer-Krebs phosphate buffer, 1 hour at 37°, total volume 1.5 cc.
Each experiment represents the average of at least three separate experiments.
* L.A.C. in cottonseed oil was used for this experiment; the controls contained an equal volume of cottonseed oil.
† Aqueous suspension of L.A.C. Cf. text.

shaking in Ringer-Krebs phosphate buffer at room temperature for one hour. Experiment 3 shows that this preparation, at a concentration equivalent to 0.05 cc. of L.A.C. in cottonseed oil, increases aerobic glycolysis from 2.0 to 4.1 without any effect on the number of stained cells.[5] With higher concentrations or longer time intervals, both L.A.C. preparations show lymphocytolysis.

In experiments designed to elucidate the mechanism by which L.A.C. increases aerobic glycolysis of lymphocytes, it was shown that certain organic and inorganic solutions of high osmolarity also produce this effect (Miller, 1953 and 1954). The influence of these solutions on the permeability to safranin has, therefore, been studied (Table 3). The addition of 140 milliosmols/liter of KCl has no effect on aerobic glycolysis, but increases the number of cells stained by safranin from 1 to 30%. On the other hand, NaCl, NH_4Cl, LiCl, mannitol and sucrose all increase both $Q_A^{O_2}$ and the permeability of the cell to safranin (Expt. 1). At 280 milliosmols/liter, all

[5] The apparently greater effectiveness of this aqueous preparation in stimulating aerobic glycolysis is not particularly significant. It is probably attributable to the variations in activity we have observed in different lots of Lipo-Adrenal Cortex, which is standardized only in terms of its hydrocortisone content. Cf. also Miller (1954).

TABLE 3. EFFECT OF HIGH OSMOLAR ENVIRONMENT ON THE AEROBIC GLYCOLYSIS AND DYE PERMEABILITY OF THYMUS LYMPHOCYTES

Addition	Experiment 1		Experiment 2	
	140 milli-osmols/liter		280 milli-osmols/liter	
	$Q_A^{O_2}$	% cells permeable to safranin	$Q_A^{O_2}$	% cells permeable to safranin
None	2.5	1	2.0	7
L.A.C.*	4.9	2	4.1	8
KCl	2.5	30	4.2	65
NaCl	3.5	24	5.9	79
NH_4Cl	5.1	13	5.9	50
LiCl†	5.7	31	5.2	69
Mannitol	5.1	35	5.5	60
Sucrose	5.8	38	9.7	66
Urea	—	—	1.9	4

* L.A.C. dissolved in Ringer-Krebs phosphate buffer, equivalent to 0.05 cc. L.A.C. in cottonseed oil. Cf. text.

† Some precipitation occurs, probably $(Li)_3PO_4$.

Ringer-Krebs phosphate buffer, M/50 glucose, 1 hour at 37° C., total volume = 1.5 cc.

the compounds studied with the exception of urea increase both $Q_A^{O_2}$ and permeability to dye (Expt. 2). Urea is completely without effect.

DISCUSSION

These experiments were originally undertaken in an effort to explain the lymphocytolytic action of L.A.C. in enzymatic terms, and, if possible, to relate this effect to increased aerobic glycolysis. We have observed marked stimulation of the aerobic glycolysis of thymus lymphocytes at concentrations of L.A.C. which produce little or no increase in the percentage of cells permeable to safranin. If these two phenomena are related, this might suggest that the primary alteration in the cell is increased glycolysis, and that this is followed by increased permeability to the dye. All the solutions of high osmolarity studied, with the exception of KCl and urea, increase both glycolysis and the percentage of safranin-stained cells. There is, however, no clear-cut correlation between the magnitude of these two phenomena. Urea is completely without effect at 280 milliosmols per liter, and increased staining of cells by safranin without increased glycolysis has been noted in the presence of KCl.

Our experiments indicate that the number of lymphocytes stained by safranin can be reduced by the addition of a non-specific protein such as bovine plasma albumin, for which the dye apparently has a high affinity. It would appear, therefore, that cells which stain after treatment with various agents do possess an increased permeability to the dye or the dye-protein complex.

Because of their relative impermeability, high concentrations of NaCl, LiCl, sucrose and mannitol probably dehydrate the lymphocyte cell. Potassium and ammonium salts, although more freely permeable, would pro-

duce the same effect at high osmolar concentrations. Increased glycolysis might result from this chain of events, for K^+ is known to stimulate phosphohexokinase, which catalyzes the trans-phosphorylation of fructose-6-phosphate into fructose diphosphate (Muntz and Hurwitz, 1951, and Muntz, 1953).

It is of considerable interest that although L.A.C. stimulates the glycolysis of glucose as much as a hundred per cent, it has little or no effect on the glycolysis of fructose diphosphate (Miller, 1954). The suggestion therefore has been made that L.A.C. stimulates the glycolysis of glucose by increasing the rate of formation of fructose diphosphate, which is frequently the limiting reaction in the dismutation of glucose to lactic acid. This might occur if L.A.C. increased the internal K^+ concentration, or the rate of entry of glucose into the cell.

The increased glycolysis produced by L.A.C. appears to be due to an alteration in the cell membrane and its permeabilify to various substances. This is further substantiated by our observation that L.A.C. stimulates the glycolysis of lymphocytes only if the cell membrane is intact, and has no effect on cell-free homogenates (Miller, 1954). This subtle change in permeability may eventually reduce the ability of the cell to exclude dyes such as eosin and safranin.

SUMMARY

1. Lipo-Adrenal Cortex increases the aerobic glycolysis of rat thymus lymphocytes before any change in the permeability to safranin occurs.

2. Glycolysis of thymus lymphocytes is also increased by high concentrations of various organic and inorganic compounds. This is accompanied by increased staining with safranin.

3. The results obtained are discussed in terms of the effect of these agents on the permeability of thymus lymphocytes.

REFERENCES

BARKER, S. B. AND W. H. SUMMERSON: *J. Biol. Chem.* **138**: 535. 1941.
DUBNOFF, J. W.: *Arch. Biochem.* **17**: 327. 1948.
FELDMAN, J.: *Endocrinology* **46**: 552. 1950.
HECHTER, O. AND S. JOHNSON: *Endocrinology* **45**: 351. 1949.
HECHTER, O. AND Z. MILLER: Unpublished.
KREBS, H. A. AND K. HENSELEIT: *Z. Physiol. Chem.* **210**: 33. 1932.
MILLER, B. F. AND J. A. MUNTZ: *J. Biol. Chem.* **126**: 413. 1938.
MILLER, Z.: *Federation Proc.* **12**: 247. 1953.
MILLER, Z.: In Press. *J. Biol. Chem.* 1954.
MUNTZ, J. A. AND J. HURWITZ: *Arch. Bioch. and Bioph.* **32**: 137. 1951.
MUNTZ, J. A.: *Arch. Bioch. and Bioph.* **42**: 435. 1953.
SCHREK, R.: *J. Cell. and Comp. Physiol.* **28**: 277. 1946a.
SCHREK, R.: *Radiology.* **46**: 395. 1946b.
SCHREK, R.: *Endocrinology.* **45**: 317. 1949.

OBSERVATIONS ON A RELATIONSHIP BETWEEN TOTAL THYROID IODINE CONTENT AND THE IODIDE-CONCENTRATING MECHANISM OF THE THYROID GLAND OF THE RAT[1]

W. P. VANDERLAAN AND RAYNOR CAPLAN

The Ziskind Research Laboratories, New England Center Hospital, and the Department of Medicine, Tufts College Medical School, Boston, Massachusetts

THE maintenance of a gradient for iodide ion between serum and thyroid parenchyma (VanderLaan and VanderLaan, 1947) has been found to be partially dependent upon the presence of thyrotropin (VanderLaan and Greer, 1950). The greatly enhanced capacity of the thyroid cell for trapping iodide under conditions of thyroid hyperplasia has generally been held a result of increased thyrotropin elaboration by the pituitary; however, recent studies (Halmi *et al.*, 1953) require revision of this concept and indicate that conditions within the thyroid gland alter the response to thyrotropin at least in regard to this function. This paper presents a further inquiry into the iodide-concentrating mechanism of the thyroid gland, especially as it is influenced by factors altering thyroid function. An inverse relationship between thyroid-bound iodine content and iodide-concentrating capacity has been observed even when stimulation by thyrotropin was at a constant level.

MATERIAL AND METHODS

Immature, albino, male rats were used. Tap water and Purina Mink Chow were given *ad libitum*. Rats were weighed at the beginning and conclusion of experiments. Chemical agents were ground in a mortar with a small quantity of the diet which was then mechanically mixed into the total diet: propylthiouracil in 0.03% concentration, potassium thiocyanate in 0.3% concentration, and potassium iodide in 0.7% concentration. The low iodine (280 micrograms iodine per kilogram) diet used consisted of the following: cracked wheat 66%, casein 15%, whole milk powder 15%, brewer's yeast, 2.5%, calcium carbonate (C.P.) 1.0%, sodium chloride (C.P.) 0.5%. To this was added 1 ml. of White's Cod Liver Oil Concentrate per kilogram of diet. In one experiment 5 mg. of KI was added per kilogram of this mixture. Both *dl*-thyroxine and thyrotropin[2]

Received for publication September 14, 1953.

[1] This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

[2] Thyrotropin, Lot #50P4, Parke, Davis and Company, assaying 2 U.S.P. units per milligram.

were injected subcutaneously. Hypophysectomy was performed by the parapharyngeal route, and its completeness was checked by inspection after the animals were killed.

Experiments were concluded by anesthetizing rats with ether and by withdrawing as much blood as possible from the abdominal aorta. Thyroid glands were dissected and weighed on a Roller-Smith torsion balance of 100 mg. capacity. Thyroid iodine content was determined by a modification of the method of Zak *et al.* (1952).

Determination of the iodide ratio between thyroid and serum was made as previously described (VanderLaan and Greer, 1950) with the modification that counting the radioiodide of serum and of thyroid was done at a 1.0 ml. volume, dispersion from thyroid tissue having been effected in chloric acid, using a Sylvania GG-306 gamma ray counter.

RESULTS

Experiment 1: This experiment was undertaken to test an hypothesis that the sensitivity of the thyroid gland to thyrotropin would be enhanced by the condition of a low thyroid iodide ion content. Accordingly, rats were hypophysectomized and given daily 1 mg. doses of thyrotropin. The pool of rats was divided, one group receiving Purina Mink Chow; another, this diet plus 0.3% KSCN; a third, this diet plus 0.7% KI; and a fourth, Mink Chow plus 0.03% propylthiouracil. Thiocyanate was used as a means of providing a low intrathyroidal iodide ion concentration and it was discontinued 24 hours before killing to allow recovery of the iodide-concentrating mechanism from thiocyanate inhibition. Propylthiouracil was used as a goitrogenic substance known to have a different mode of action from thiocyanate. Ten daily injections of thyrotropin were given, and on the eleventh day the experiment was concluded. The results are shown in Table 1 and a graphical presentation is made in Figure 1. It is seen that as the dietary iodide available to the thyroid decreased there occurred an increased thyroid:serum iodide ratio. Thus, the diet with added KI, Mink Chow alone, and Mink Chow with thiocyanate, in decreasing order of availability of iodide ion, resulted in an increasing capacity of the thyroid parenchyma to pump iodide ion from the serum. However, the figure indicates that under all conditions promoting a decreased total thyroid iodine content, i.e. bound iodine, which includes hormonal iodine, there is associated a correspondingly increased iodide ion gradient regardless of the availability of

TABLE 1. THE EFFECT OF EXPERIMENTAL DIETS ON THYROID:SERUM IODIDE RATIO, THYROID IODINE CONCENTRATION, AND THYROID WEIGHT IN HYPOPHYSECTOMIZED RATS RECEIVING 1 MG. OF THYROTROPIN DAILY FOR TEN DAYS

Diet	Thyroid:serum iodide ratio	Thyroid iodine mg./100 gm. thyroid	Thyroid wt. mg./100 gm. rat
Control	25.4 ± 5.5*	34.4±3.0*	13.4±0.2*
+0.7% KI	5.93± 1.95	43.2±8.4	14.6±0.9
+0.3% KSCN	49.1 ± 6.3	29.0±3.1	12.9±0.85
+0.03% Propylthiouracil	108.4 ±10.7	6.8±0.96	12.6±0.7

* Standard error of the mean.

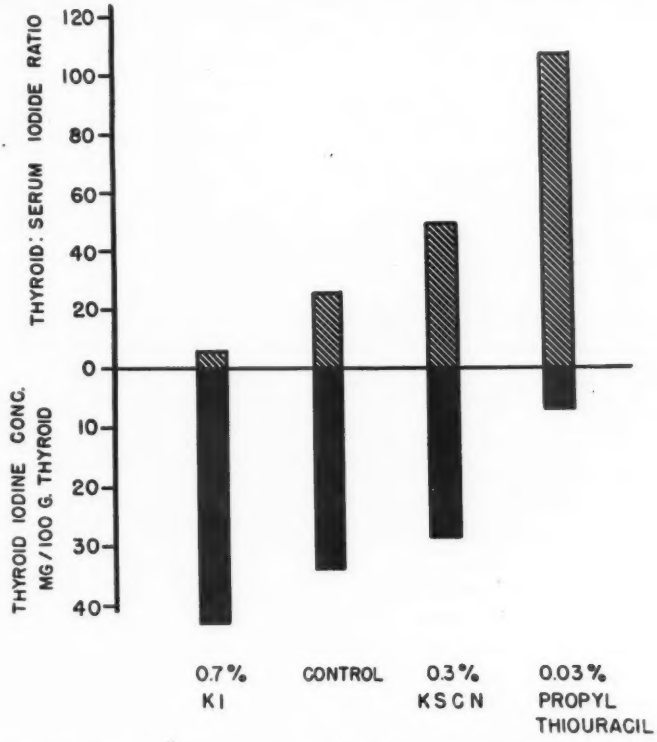


FIG. 1. Rats were hypophysectomized, fed the experimental diet, and injected with 1 mg. thyrotropin daily for 10 days. Thiocyanate was omitted the last 24 hours. A reciprocal relationship between total thyroid iodine content and iodide gradient is seen.

iodide to the thyroid. At this time the prior experiments of Halmi *et al.* (1953) were published, and it was apparent that the present experiments were confirmatory of theirs and, in some regards, were in extension of them. In addition, however, the results required abandonment of the premise of the experiment in that propylthiouracil had the most marked effect on the iodide gradient, although it is known not to depend for its antithyroid effect on a diminution of the capacity of the thyroid cell to concentrate iodide ion (Astwood, 1944–45). It is further apparent that the gradient was greatly enhanced under conditions in which the thyroid weight was not changed. Finally, under the conditions of the experiment the effect of thyrotropin on thyroid weight was not altered by either concurrent iodine or propylthiouracil treatment; this is in contrast to the experience of Albert *et al.* (1947) in chicks but agrees with the observations of Astwood (1944–45).

TABLE 2. THE EFFECT OF THE ADDITION OF A SMALL INCREMENT OF IODINE TO A DIET LOW IN IODINE ON THYROID:SERUM IODIDE RATIO, THYROID IODINE CONCENTRATION, AND THYROID WEIGHT IN HYPOPHYSECTOMIZED RATS RECEIVING 1 MG. OF THYROTROPIN DAILY FOR SIX DAYS

Diet	Thyroid:serum iodide ratio	Thyroid iodine mg./100 gm. thyroid	Thyroid wt. mg./100 gm. rat
Low Iodine	58.7 ± 6.3*	16.1 ± 2.5*	15.4 ± 1.2*
Low Iodine + 5 mg. KI per kilo of diet	21.5 ± 3.0	26.8 ± 5.1	15.0 ± 0.9

* Standard error of the mean.

Experiment 2: The total thyroid iodine concentration was manipulated by feeding one group of hypophysectomized rats the low iodine diet and a second group this diet to which 5 mg. of KI per kilogram of diet was added. Thyrotropin was given daily for 6 days, and the thyroid weight, iodide gradient, and iodine content were determined. The data are given in Table 2 and indicate that the diet low in iodine resulted in thyroid glands of equal weight, lower iodine content, and almost three-fold increased avidity for iodide, when compared with glands from rats receiving this ration with added iodide.

Experiment 3: The previous two experiments having indicated an association between iodine content of the thyroid gland and the capacity for iodide accumulation using as test animals hypophysectomized rats receiving constant doses of thyrotropin, interest attached to the relationships in time of changes in iodine content and iodide gradient in intact rats administered propylthiouracil. Accordingly, intact rats were injected intraperitoneally with 10 mg. of propylthiouracil, and the substance was added to the diet at this time. The pool of rats was sampled 0, 16.5, 30, 45, and 64 hours after the injection of propylthiouracil, and thyroid weight, total iodine content, and iodide gradient were determined. The results are presented in Figure 2 and indicate that in intact rats 30 hours and thereafter following administration of propylthiouracil there was a rapidly rising gradient, which was associated with a falling thyroid iodine content. At 16.5 hours neither iodine content nor iodide gradient changed significantly and throughout the experiment thyroid weight was constant in all groups.

Experiment 4: The modification by thyroxine of thyroid weight, iodine content, and iodide gradient in intact rats continuing to receive propylthiouracil was studied in this experiment. In view of the preceding experiments observations were made on the thyroid weight, total iodine content, and iodide gradient of rats previously chronically treated with propylthiouracil, the latter being continued during the period of thyroxine injections. Figure 3 shows that the daily injection of 20γ *dl*-thyroxine under these conditions resulted in a rapid decline of iodide gradient in the first

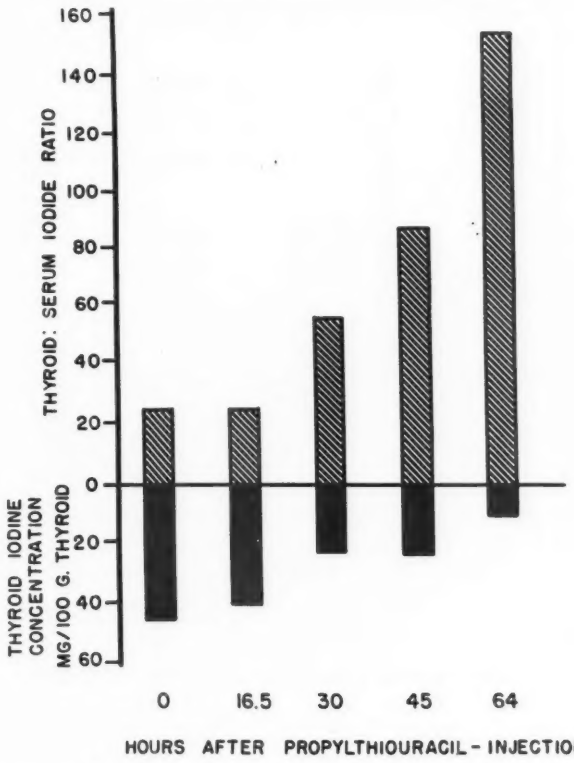


FIG. 2. Intact rats were administered propylthiouracil and the effect on thyroid iodine content and thyroid:serum iodide ratios was observed periodically thereafter. Iodide ratio mounts rapidly as total thyroid iodine content declines.

four days and a more gradual decline thereafter until a normal value of 25:1 was reached at 10 days. Thyroid weight declined similarly, but little effect was observed in thyroid iodine concentration. It appears likely that this dose of thyroxine resulted in marked suppression of pituitary thyrotropin formation, and that complete suppression by thyroxine of the thyroidal capacity to accumulate iodide requires very large doses or prolonged administration if conditions which produce a continuing low iodine content of the thyroid gland are maintained.

Experiment 5: The effect on thyroid:serum iodide ratios of feeding propylthiouracil to rats before and after hypophysectomy with and without thyrotropin stimulation was studied. Hypophysectomy was performed on rats, half of which had received a normal diet, and the remainder 0.03% propylthiouracil for at least 10 days. After hypophysectomy each group

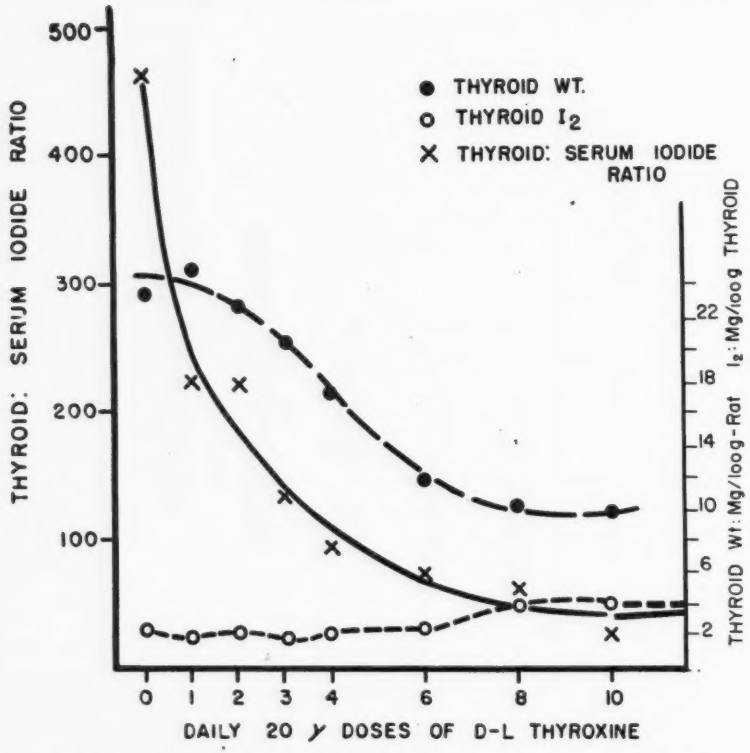


FIG. 3. Intact rats were treated with propylthiouracil for 10 days before daily injections of 20γ of *dl*-thyroxine were begun. Propylthiouracil was continued throughout. Thyroxine causes an initial rapid decline in iodide ratio and thyroid weight. The total thyroid iodine content changes little. Suppression of iodide gradient below normal did not occur in the 10 days studied.

was subdivided, propylthiouracil being fed to half of the subgroup and plain mink chow to the other half, four combinations resulting. Five days after hypophysectomy thyroid weight, iodine content, and iodide gradient were determined. Four other groups of rats were prepared similarly, and thyrotropin in daily 1 mg. doses was given beginning 6 days after hypophysectomy.

From Table 3 and Figure 4, in which the results are recorded, it is clear that 5 days after hypophysectomy and in the absence of thyrotropin injections there generally existed an inverse relationship between total thyroid iodine content and the gradient for iodide. The lowest values for this gradient were found in rats with normal thyroid iodine contents, those not

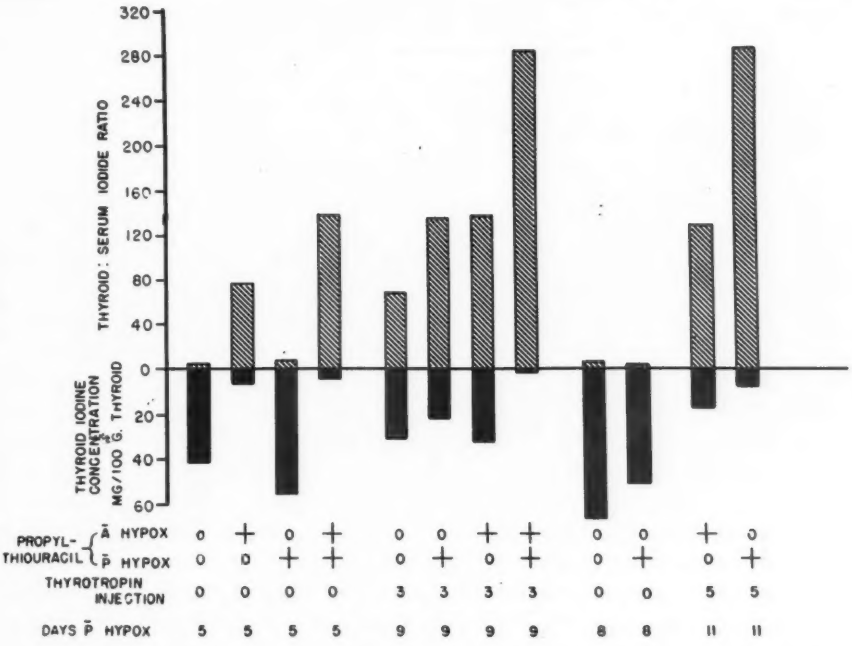


FIG. 4. Propylthiouracil-treated and control rats were hypophysectomized and propylthiouracil was given to half of each group. Thyrotropin was given to similar groups. The lowest iodide gradient is observed in untreated hypophysectomized rats, the highest in thyrotropin-treated and propylthiouracil-treated rats. Generally a reciprocal relationship between iodine content and iodide gradient is observed. After hypophysectomy propylthiouracil has little effect on iodine content or thyroid:serum iodide ratio. Propylthiouracil administration before hypophysectomy is indicated by $\bar{A}$ Hypox, after hypophysectomy by $\bar{P}$ Hypox.

given propylthiouracil before hypophysectomy. In the experiment in which thyrotropin was not given the highest iodide ratios occurred in the group receiving propylthiouracil both before and after hypophysectomy, and a very low total thyroid iodine content was found.

Similarly in those rats receiving thyrotropin after hypophysectomy the highest iodide gradient occurred in rats treated with propylthiouracil, and again an inverse relationship between iodine content and iodide gradient is to be observed. It is noteworthy, however, that an iodide ratio of 139 was found 9 days after both hypophysectomy and the discontinuation of proplythiouracil in the group which received three daily injections of thyrotropin before being killed. A moderate suppression of iodine content was noted.

TABLE 3. THE EFFECT OF FEEDING PROPYLTHIOURACIL BEFORE AND AFTER HYPOPHYSECTOMY AND WITH AND WITHOUT THYROTROPIN ON THYROID WEIGHT, THYROID IODINE CONTENT, AND THYROID:SERUM IODIDE RATIO

Propylthiouracil		Daily injections* Thyrotropin	No. days after hypophysectomy to Sacrifice	Thyroid wt.: mg./100 gm. rat	Iodine content: mg./100 gm. thyroid	Thyroid:serum iodide ratio
Before Hypophysectomy	After hypophysectomy					
0	0	0	5	14.5±0.96†	41.5 ± 5.6†	4.24± 0.83†
+	0	0	5	20.4±3.0	6.47± 0.56	77.9 ± 8.0
0	+	0	5	11.5±1.4	54.7 ± 1.6	7.59± 0.91
+	+	0	5	17.5±2.2	3.63± 0.40	139.1 ±20.9
0	0	3	9	12.4±0.7	30.5 ± 4.7	70.0 ± 9.7
+	0	3	9	16.7 1.0	32.1 ± 6.1	139 ±17.6
0	+	3	9	12.8±0.8	21.4 ± 4.9	136.3 ±21.3
+	+	3	9	17.3±0.8	1.93± 0.13	285 ±77
0	0	0	8	9.6±0.6	66.5 ±13.5	8.69± 3.56
0	+	0	8	12.4±1.2	50.5 ± 7.8	5.62± 2.02
+	0	5	11	14.8±1.4	17.8 ± 3.1	130.1 ±43.0
0	+	5	11	11.8±0.3	7.4 ± 0.7	288 ±54.0

* Thyrotropin initially injected six days after hypophysectomy.
† Standard error of the mean.

DISCUSSION

The experiments on the regulation of the iodide concentrating mechanism of the thyroid gland not only indicate that this function is influenced by thyrotropin, but more importantly confirm the observations of Halmi *et al.* (1953) that propylthiouracil administration to hypophysectomized rats greatly enhances the effect of thyrotropin on this function of the thyroid gland. Further, it is notable that other measures in addition to propylthiouracil treatment produce this effect, particularly withdrawal of potassium thiocyanate, and that this function in hypophysectomized rats receiving thyrotropin is altered by the iodine concentration of the diet. In general, by a variety of experiments it has been found that there is a reciprocal relationship between the total iodine content of the thyroid gland and its capacity for concentrating iodide ion. In all instances but the one in which KI was added to the diet in 0.7% concentration, it seems accurate to assume that the total iodine content of the thyroid was almost entirely comprised of protein-bound iodine, and hence an indication, but not a measure of the hormonal iodine content. This reciprocal relationship has been observed in intact rats and in hypophysectomized rats. Figure 3 may seem an exception to this, but throughout the iodide ratio is high considering the amount of thyroxine administered. Highest concentration gradients for iodide were observed only in the presence of the pituitary or after thyrotropin administration. However, 5 days after hypophysectomy in rats not given thyrotropin a five- to six- fold increase in iodide gradient over normal was observed in thyroid glands of low iodine content, indicating a measure of autonomy of this function of the thyroid gland.

The present data are of interest in regard to self-regulation of iodine

metabolism by the thyroid gland. They indicate that an increased elaboration of thyrotropin is not necessary for an enhanced thyroidal capacity to accumulate iodide ion, but rather that iodide-pumping is enhanced by a variety of circumstances associated with lowered total thyroidal iodine content. Previously, VanderLaan and VanderLaan (1947) showed that the gradient for iodide between thyroid and serum remains constant over a wide range of serum iodide concentrations. Morton, Chaikoff, and Rosenfeld (1944), studying sheep thyroid slices *in vitro*, found increasing iodide concentration in the medium to be associated with increased thyroxine formation, although inhibition was observed at high iodide levels. Raben (1949) found that iodine administration, below levels inhibiting thyroid function, was associated with an increased rate of hormone formation. Stanley (1949) confirmed this in man, finding no depression of the rate of radioactive iodine accumulation when small increments of I^{127} were injected intravenously during the period of study. Below inhibitory levels, therefore, added iodide increases markedly the thyroid iodide content and, in turn, increases the rate of hormone formation. Since the data presented here indicate that circumstances lowering thyroidal bound iodine are associated with an enhanced efficiency of the gland in pumping iodide, it is apparent from the foregoing that this tends to restore toward normal the original deficit of thyroid bound iodine, so that a mechanism exists within the thyroid gland for the regulation of iodine metabolism which does not depend on fluctuations in thyrotropin stimulation. To this mechanism can reasonably be attributed the phenomenon that the total iodine content of the thyroid remains constant, as observed from one rat to another under reasonably normal environmental and dietary conditions, an observation hardly explained on the basis of pituitary regulation. A pituitary regulatory mechanism of thyroidal function sensitive to bodily needs and to the level of circulating thyroid hormone is clearly implied by available data, whereas the mechanism by which the pituitary, simply speaking, could assess the iodine stores of the thyroid gland appears inscrutable.

Restated, it appears that the iodide ion pump of the thyroid gland is activated in association with conditions decreasing thyroidal hormonal stores and, conversely, it is inactivated with conditions increasing thyroidal hormonal stores. These variations do not depend entirely on the pituitary. These data may bear on the problem of Graves' disease in regard to the fact that low hormonal stores are characteristic of the disorder and a high iodide concentration gradient is observed. The association of the two according to the present data need not be a result of continuing excessive thyrotropic hormone stimulation, but rather may aid in themselves in the perpetuation of high hormone output. It is a matter of speculation that since iodide ion administration in Graves' disease results in increase in

bound iodine stores in the gland, the consequent lowering of the iodide gradient could result in decreased hormone formation. This speculation would help explain the mode of action of iodine in Graves' disease only if the lowering of the gradient proves disproportionally greater than the rise in blood iodide levels.

Chapman's data (1941) warranted the contention that the thyroid gland had a degree of autonomy in that he found a low iodine content of the diet enhanced thyroid gland size and cell height in hypophysectomized animals. Thus, evidence anatomically derived indicates also that there is a measure of self-regulation of the thyroid gland.

The data presented are in keeping with those of Halmi *et al.* (1953) that led them to reject Greer's hypothesis (1952) of two thyrotropins. Wide variations in thyroid:serum iodide ratios have been observed in rats with constant thyroid gland size, indicating the former to be the more sensitive indicator of thyrotropin activity. Only one thyrotropin need be postulated at this time according to the present data.

Thyroxine in doses in excess of normal endogenous output by several fold returned the iodide gradient to normal only after ten days under the conditions of the experiment. Interpretation of this is difficult. Halmi *et al.* (1953) concluded that thyroxine is more efficient than hypophysectomy in reducing the iodide gradient. The dose used by them, 200 μg. thyroxine daily for 20 days, carried such a large increment of iodine that the interpretation of its effect would be facilitated by a control experiment in which iodine itself was administered in corresponding doses. This reservation appears to find support in another observation by them that "a depressing effect of thyroxine on the T/S response to TSH could thus not be demonstrated under the conditions of this experiment." In the latter instance 20 μg. doses of thyroxine in addition to the thyrotropin were used.

SUMMARY AND CONCLUSIONS

In hypophysectomized rats receiving thyrotropin in constant doses, the capacity of the thyroid gland to accumulate iodide ion has been found to be enhanced by measures which have in common a tendency to lower the total thyroidal iodine concentration. These include iodine restriction and treatment with propylthiouracil and with potassium thiocyanate. In the instance of thiocyanate the drug was withdrawn 24 hours before thyroid:serum iodide ratios were measured to allow recovery of the concentrating mechanism from inhibition. A similar reciprocal relationship between iodide-concentrating capacity and total thyroidal iodine concentration was found in intact animals. A measure of self-regulation of the thyroid gland in iodine metabolism has been postulated, and the significance of this in

regard to normal thyroid function and thyroid function in Graves' disease, especially as it is modified by iodine administration, has been discussed.

Data have been presented which indicate that thyroxine has a marked effect on iodide-concentrating capacity. However, it has been held that contentions that thyroxine is more effective in lowering this iodide gradient than hypophysectomy are not fully warranted by the data thus far presented. Evidence in support of the concept that iodide-concentrating capacity is a more sensitive indicator of thyrotropin activity than thyroid weight has been presented, and it has been held unnecessary to hypothesize two thyrotropins.

Acknowledgments

The authors are indebted to Dr. E. B. Astwood for advice, criticism, and the privilege to work in his laboratory.

Thyrotropin was supplied through the kindness of Dr. D. A. McGinty, Parke, Davis & Company, Detroit.

REFERENCES

ALBERT, A., R. W. RAWSON, C. RIDDELL, P. MERRILL AND B. LENNON: *Endocrinology* **40**: 361. 1947.

ASTWOOD, E. B.: *The Harvey Lectures* **40**: 195. 1944–45.

CHAPMAN, A.: *Endocrinology* **29**: 681. 1941.

GREER, M. A.: *J. Clin. Endocrinol. and Metab.* **12**: 1259. 1952.

HALMI, N. S., B. N. SPIRTOS, E. M. BOGDANOVE AND H. J. LIPNER: *Endocrinology* **52**: 19. 1953.

MORTON, M. E., I. L. CHAIKOFF AND S. ROSENFELD: *J. Biol. Chem.* **154**: 381. 1944.

RABEN, M. S.: *Endocrinology* **45**: 296. 1949.

STANLEY, M. M.: *J. Clin. Endocrinol.* **9**: 941. 1949.

VANDERLAAN, J. E. AND W. P. VANDERLAAN: *Endocrinology* **40**: 403. 1947.

VANDERLAAN, W. P. AND M. A. GREER: *Endocrinology* **47**: 36. 1950.

ZAK, B., H. H. WILLARD, G. B. MYERS AND A. J. BOYLE: *Anal. Chem.* **24**: 1345. 1952.

THE ENDOMETRIAL CUPS AND ALLANTOCHORIONIC POUCHES IN THE MARE WITH EMPHASIS ON THE SOURCE OF EQUINE GONADOTROPHIN

M. T. CLEGG, J. M. BODA AND H. H. COLE

Department of Animal Husbandry, University of California, Davis, California

CATCHPOLE and Lyons (1934) were the first to suggest the placenta as the source of the gonadotrophin appearing in the blood of pregnant mares. They concluded from biological and histological studies that the chorionic epithelium was the most likely source of the hormone. Cole and Goss (1943) on the other hand, concluded that the hormone was produced by specialized endometrial structures referred to as endometrial cups. They explained any apparent gonadotrophic activity of the chorion on the basis of a sticky material secreted by the cups and readily adhering to the chorion. Schauder (1912) described these cups and speculated that they were the precursors of the "pendulous hippomanes." The "pendulous hippomanes" of Schauder will be referred to as allantochorionic pouches. Schauder concluded that the endometrial cups (Schleimhautkrater) invariably present at a certain stage of pregnancy, should not be considered as pathological but, rather, as normal structures having a physiological function to perform.

It is the purpose of the present paper to consider:

1. The stimulus for the formation of the endometrial cups.
2. Further evidence on the source of equine gonadotrophin: the relationship between the development of the cups and the initial appearance of gonadotrophin in the blood; the specific cells concerned with the secretion of the hormone.
3. The duration of activity and fate of the endometrial cups in relation to the disappearance of gonadotrophin in the blood.
4. The development and fate of the allantochorionic pouches.
5. The amount of gonadotrophin in the cups and allantochorionic pouches at different stages of pregnancy.

MATERIALS AND METHODS

In 1949 and 1950, tissues were collected from 108 pregnant mares with fetuses ranging in crown-rump length from 2.2 to 81.3 cm. The type of mare has been described earlier (Cole, Howell and Hart, 1931).

Received for publication September 28, 1953.

The methods of collection and treatment were somewhat different for the two years. In 1949, the endometrial cup secretion was separated from the endometrial cups. No attempt was made to quantitatively separate the cups and secretions as was done by Cole and Goss, 1943, and thus the values for hormonal contents of the endometrial cups are high because of the greater amount of residual secretion present. Both cups and the secretion were frozen with dry ice at once and kept in a frozen state for two weeks. After thawing, the secretion was mixed with a small amount of saline and then acetone was added to 30% by volume. The secretion was stored in this condition—in some instances for two months before assaying. The endometrial cups were likewise placed in a small amount of saline after thawing and then triturated in a Waring blender. Acetone was added to 30% by volume for storage.

In 1950, to obviate difficulty encountered in handling the sticky secretion, the endometrial cups, secretion and the overlying allantochorion were clipped off together with scissors and frozen with dry ice. This mass was stored for two to three weeks in the frozen state. Then it was placed in 10% acetone in saline solution, ground in a Waring blender and stored until tested. Much higher values for total hormone recovered in individual

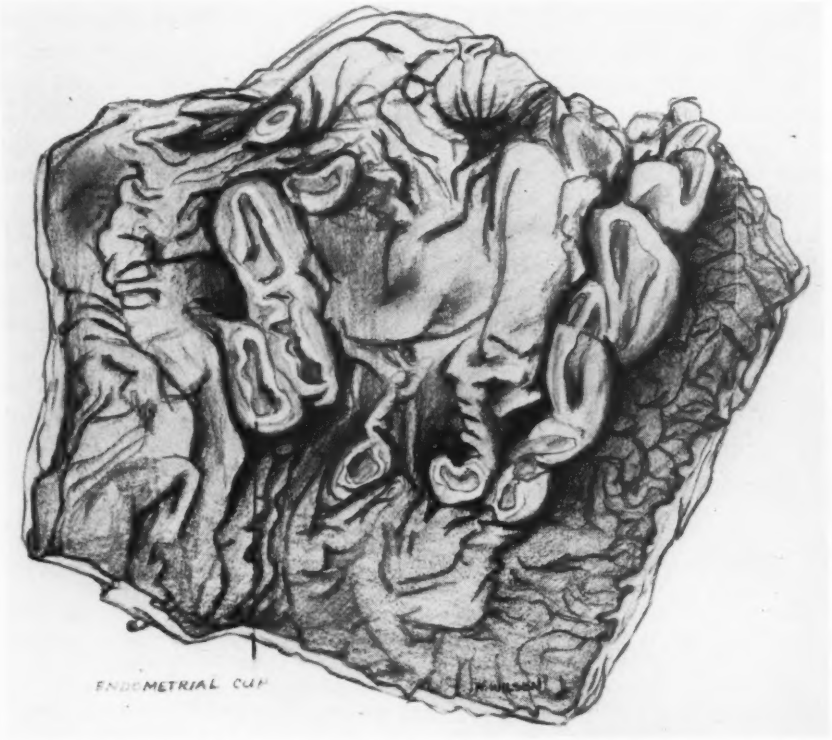


FIG. 1. Drawing of entire area of fully developed endometrial cups. Edges of cups easily distinguished from surrounding folds of uterine mucosa. Mare 456 Fetal crown-rump length (C.R.) 9.0 cm. ×1.

cases were obtained in 1949 than for 1950, but more recent studies in which the methods were compared indicate that this greater yield did not depend upon the difference in treatment.

During both 1949 and 1950, one endometrial cup from each mare was fixed in Bouin's or Carnoy's. After sectioning in paraffin, the tissues were stained with hematoxylin and eosin or with Feulgen reagent after oxidation by means of periodic acid (McManus, 1946). The only modification of the McManus method was that the tissues were counterstained with light green—S.F., an acid dye.

Gonadotrophin assays were made according to the method of Cole and Erway (1941).

RESULTS

The stimulus for the formation of the endometrial cups. The endometrial cups invariably form in that portion of the endometrium in apposition to the chorion in the region where the allantoic blood vessels fan out over the allantochorion from the umbilical vessels (Fig. 2). The constancy of this relationship strongly suggests that some substance is diffusing from the allantochorion and possibly from the allantoic vessels to act as a stimulus for the development of the endometrial cups. If this were not true, one might except the cups to form haphazardly over the endometrium in contact with the chorion.

Evidence on the source of gonadotrophin. If the endometrial cups are the source of the gonadotrophin in the blood of pregnant mares, a relationship should exist between the appearance of gonadotrophin in the blood and the development of the cups. The studies of Catchpole and Lyons (1934) and of Cole *et al.* 1931) showed that the hormone enters the blood shortly after the fetus attains a crown-rump length of 2.0 cm. The endometrial cups have been studied and the hormonal content of the blood determined for several mares at approximately this stage of pregnancy. In the four mares, having a hormonal concentration of 11 I.U. or less per cc. of blood serum (Table 1) the endometrial cups are just beginning to develop; in fact, the cups have not assumed their characteristic form but consist merely of

---

→

EXPLANATION OF PLATE 1

FIG. 2. Photograph showing allantochorionic pouches. Uterine folds between pouches evident through transparent allantochorion. In this case, the endometrial cups are desquamated and enclosed within the pouches. Mare 440 C.R. 23.3 cm. ×⅔.

FIGS. 3 and 4. Drawing of essentially the same view emphasizing variations in form of allantochorionic pouches. Mare 440 C. R. 23.3 cm. and Mare 432 C.R. 25.0 cm., respectively. ×⅔.

Abbreviations: Figs. 2–12. a.c.—allantochorion; e.c.s.—endometrial cup secretion; u.g.—uterine gland; b.v. blood vessel; e.n. endometrium outside of cup area; a.c.p.—allantochorionic pouch; u.v.—umbilical vessels; u.g.s.—uterine gland secretion.

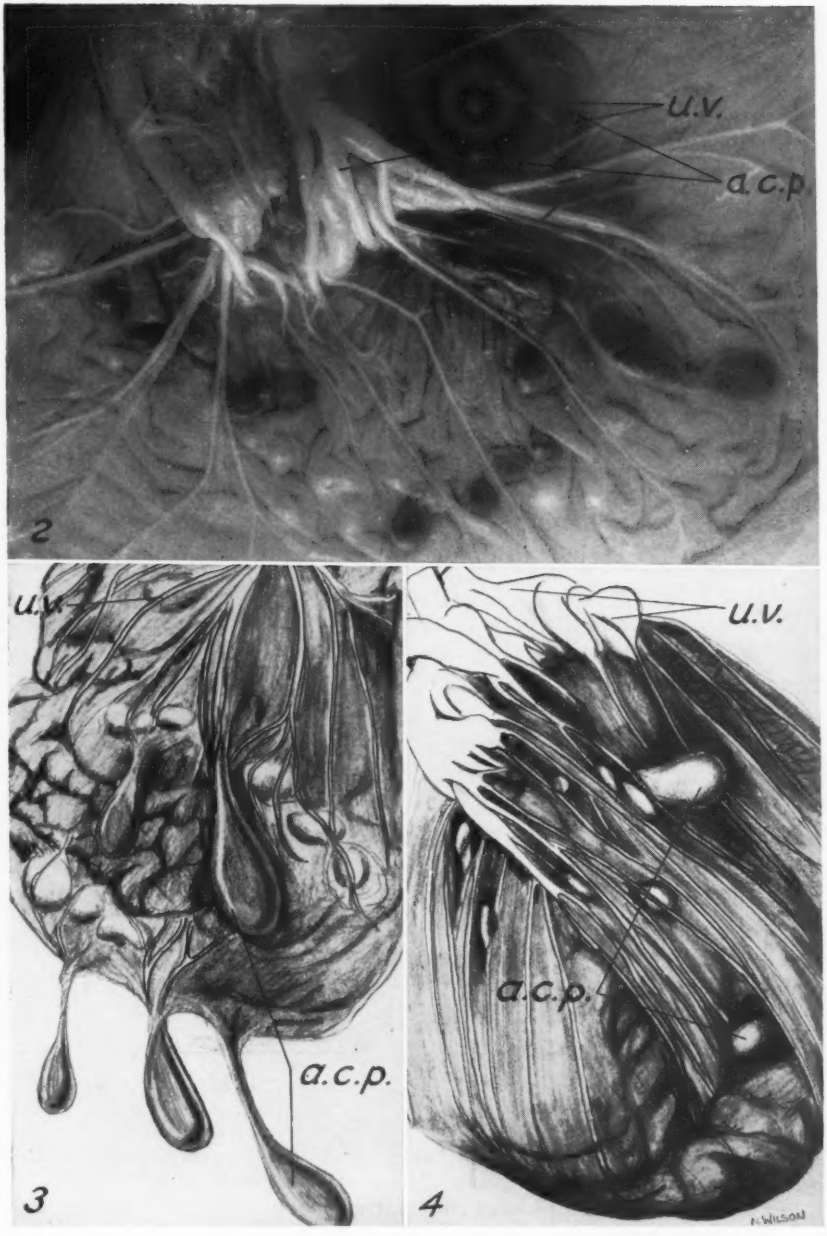


PLATE 1

modified folds. Grossly, these folds are distinguished from the other uterine folds by their pale, anemic appearance. Furthermore, these modified folds, which are the precursors of the cups, are slightly more swollen than the other uterine folds. Histologically, their differentiation from the surrounding endometrium is clear-cut (Figs. 5 and 7). The epithelium lining the uterine lumen and the necks of the uterine glands becomes stratified and the cells are polygonal rather than columnar. Especially conspicuous is the enlargement of the necks of the uterine glands. The vascularity of this area is reduced. These changes in the epithelial cells apparently precede changes in the cells of the stroma. For instance, in mare 420 with a crown rump length of 2.3 cm., changes in the epithelium in the necks of the uterine glands are well-advanced but none of the typical "decidual-like" cells are present. In slightly more advanced cases, stromal cells become enlarged and densely packed. Cole and Goss (1943) have illustrated these cells and referred to them as "decidual-like." In the sense that it is eventually desquamated, the entire mucosa in the cup region should be considered as decidual, but Mossman (1937) states "a fair definition of human decidual cells would state that they are endometrial connective tissue cells which have become rounded or polyhedral due to storage of glycogen or lipoids in their cytoplasm."

To emphasize that the cups in these early stages of pregnancy are just beginning development, a fully developed cup is shown for comparison (compare Figs. 5 and 6). Note that the changes in both the uterine glands and connective tissue cells have advanced markedly.

Before taking up the hormonal concentrations of these altered uterine folds, the precursors of the endometrial cups, let us speculate how these folds are transformed into the typical cup-like structures illustrated in Figure 1. The histological changes in these uterine folds have been described above. The evidence suggests that the central part of the fold, following the histological transformation, ceases to grow. On the contrary, there appears to be a rapid growth at the edge of this transformed area. Thus the edge is raised to form the lip of the cup.

It has been stated that the cups begin their development at approximately the time that gonadotrophin enters the blood stream. Next, the

---

→

EXPLANATION OF PLATE 2

FIG. 5. Early developing endometrial cup. Necks of uterine glands enlarged, little secretion evident. Mare 418 C.R. 2.6 cm. Haematoxylin and eosin, ×24.

FIG. 6. Fully developed endometrial cup. Secretion pouring from uterine glands. Compare size and activity of glands within cup area to glands outside. Mare 384 C.R. 6.2 cm. Haematoxylin and eosin, ×24.

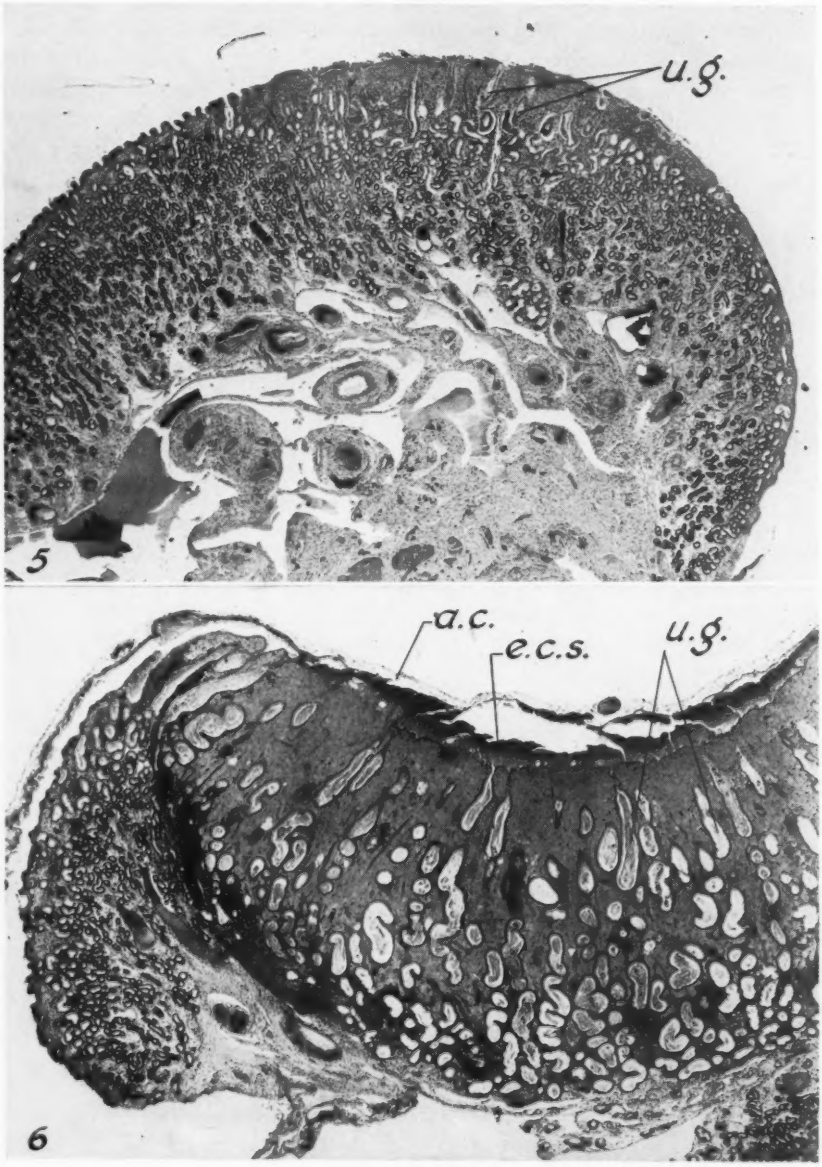


PLATE 2

hormonal concentration in the blood and in these developing cups will be compared. The tissues from the same mares used in describing the developing cups were used for comparison (Table 1). It may be seen that the cups at this early stage contain a fairly high concentration of hormone. The uterine mucosa outside the cup area is devoid of hormone. The value of 100 to 1700 I.U. of gonadotrophin per gram of endometrial cups compares with a maximum figure of 13,000 I.U. per gram found later in pregnancy.

TABLE 1. GONADOTROPHIN IN BLOOD SERUM AND IN ENDOMETRIAL CUPS AT EARLY STAGES OF PREGNANCY
(International Units per gram)

Mare number	Crown-rump length of fetus	Blood serum	Endometrial cups
	cm.		
420	2.3	3	100
405	2.4	6	1700
418	2.6	0.1	100
409	3.7	11	1700

The view that the cups are the source of equine gonadotrophin, therefore, is strengthened by three facts: The development of these cups is coincident with the appearance of hormone in the blood. These cups in early stages of development contain larger concentrations of hormone than is found in the blood, and thirdly, the uterine mucosa contains gonadotrophin only in the cup area.

No appreciable amount of endometrial cup secretion is present at this early stage of pregnancy. It would appear that, in these early cases, the endometrial cups are acting mainly as endocrine glands—in other words, most of the hormone is entering the blood stream. In mare 420 with a fetus measuring 2.3 cm. for example, a total of 300 I.U. (2994 mg. assaying 0.1 I.U. per mg.) was present in the endometrial cups and secretion whereas the blood contained 3 I.U. per cc. or a total of approximately 60,000 I.U. In other words, the blood contained 200 times the amount found in the cups and secretion substantiating the view of intensive endocrine activity. Later, the gland is exocrine as well and pours a considerable amount of the

»»→

EXPLANATION OF PLATE 3

FIG. 7. Higher magnification of Figure 5 illustrating densely packed stromal cells which have not hypertrophied as seen in later stages. Uterine gland epithelium hypertrophied and necks of uterine glands widened. To extreme left, uterine glands outside of cup area are shown. Mare 418 C.R. 2.6 cm. Haematoxylin and eosin, ×135.

FIG. 8. Fully developed endometrial cup. Uterine glands greatly enlarged and lumens filled with secretion. Note "decidual" cells, many with vacuoles. Mare 417 C.R. 7.5 cm. Mallory-Azan. ×135.

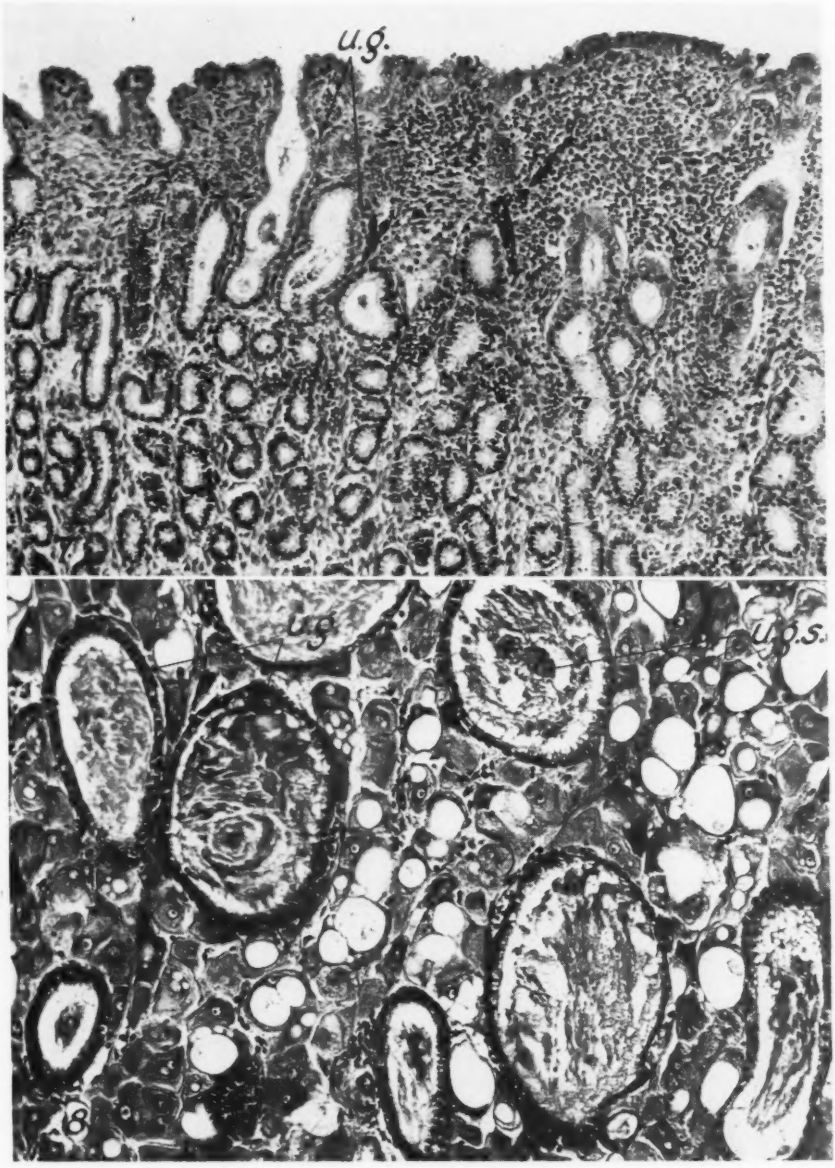
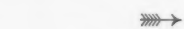


PLATE 3

hormone into the lumens of the uterine glands from which it is emptied into the space between the chorion and the endometrium. In some mares more than a million I.U. are found in the endometrial cup secretion. This compares with a maximal blood content of approximately 3 million I.U.

Cells of the endometrial cups secreting gonadotrophin. Attention has been drawn above to the fact that both the epithelial and the stromal cells in the cup area undergo marked changes. Catchpole and Lyons (1934) noted changes in the chorion indicating secretory activity. Thus, the question arises as to which cells are producing the hormone. As the hormone is a glycoprotein, we have attempted to obtain information on the cells secreting the hormone by staining with Feulgen reagent following oxidation with periodic acid (McManus, 1946). The chorion cells were not stained with leucofuchsin. The "decidual" cells of the stroma contain granules staining faintly. The epithelium lining the uterine lumen and the epithelium of the uterine glands in the cup area, on the other hand, stain intensively. This is true at the earliest stage at which hormone appears in the blood (Figs. 9 and 10). The epithelium outside the cup area is devoid of stain (Fig. 12).

The complete lack of stain in the chorionic cells does not support the concept that gonadotrophin is produced here. Mare 420 (crown-rump fetal length 2.3 cm.) is particularly vital with regard to the epithelial cells of the cup area. In this one case, the "decidual" cells have not formed. The uterine gland epithelium in the cup area, however, stains intensively with leucofuchsin, the cells showing typical apocrine secretory activity. The endometrial cups in this mare contained 100 I.U. per gram of tissue. Thus it appears that hormone may be present in the absence of "decidual" cells. This, we feel, gives strong evidence that the epithelial cells of the endometrial cup are secreting the hormone. Of interest in this one case is the presence of undifferentiated mesenchymal cells within the endometrial stroma of the cup area. They contain positively staining glycoprotein granules.



EXPLANATION OF PLATE 4

FIG. 9. Uterine gland epithelium and uterine secretion reacts positively to leucofuchsin stain. Chorionic epithelium negative. Mare 401 C.R. 5.7 cm. McManus, ×135.

FIG. 10. Uterine gland epithelium shows apocrine secretion. Granules stained red indicating glycoprotein. Secretion pouring into space between endometrium and allantochorion. Mare 419 C.R. 4.0 cm. McManus, ×275.

FIG. 11. Tips of uterine gland epithelium react to glycoprotein stain. Note positive staining of blood vessels. Mare 401 C.R. 5.7 cm. McManus, ×135.

FIG. 12. Uterine glands of endometrial cup filled with glycoprotein staining material. Glycoprotein absent in uterine glands outside of cup area. Mare 388 C.R. 7.2 cm. McManus, ×24.

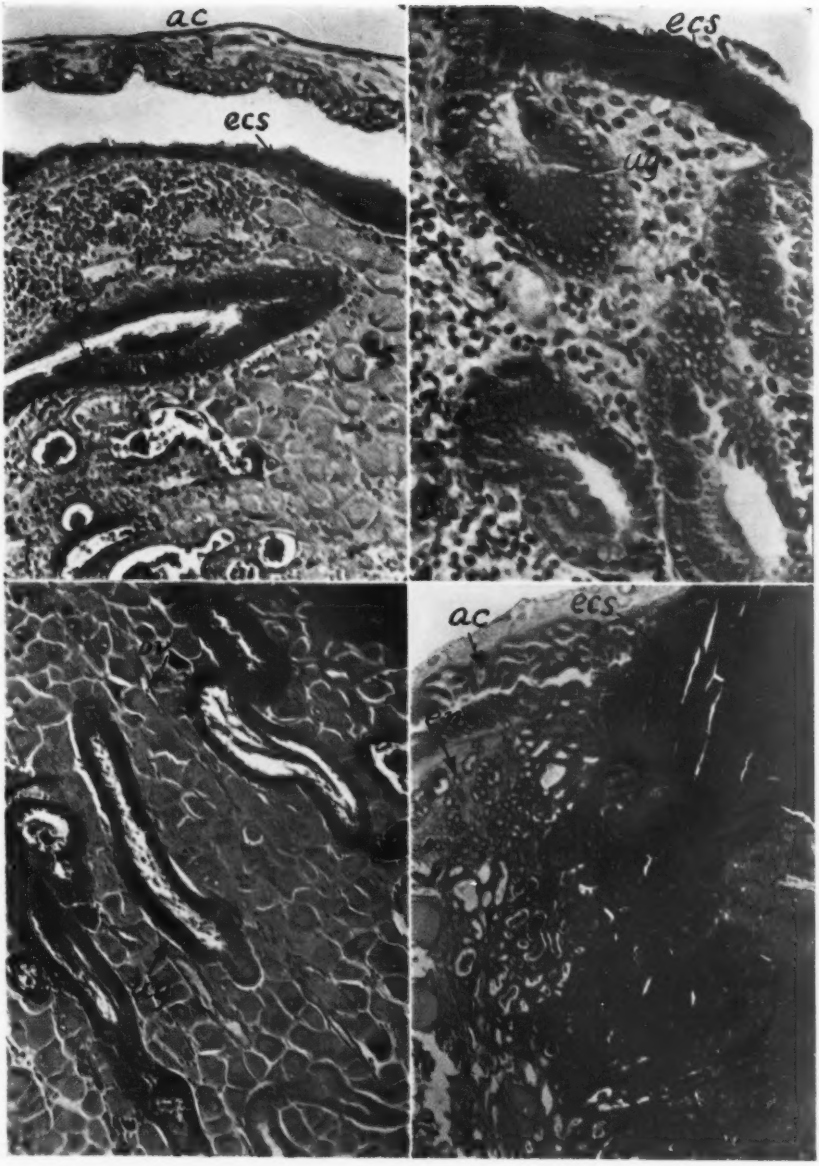


PLATE 4

FIG. 9. Upper left
FIG. 10. Upper right
FIG. 11. Lower left
FIG. 12. Lower right

Inasmuch as these mesenchymal cells were found in only this one case, it is doubtful if they play any role in gonadotrophin formation.

A complication in the interpretation of the specific staining of the epithelial cells is that mucin is likewise a glycoprotein and one would expect the uterine glands to secrete mucin. The extremely high concentration of gonadotrophin in the endometrial cups, however, is of particular significance in this interpretation. If cells other than epithelial were involved they should have reacted to the stain.

In the later stages of the developing cup when large amounts of secretion are present, the walls of the blood vessels in the cup area react strongly to the leucofuchsin stain, while the vessels in the earlier stages when little secretion is present are devoid of stain or stain only weakly (Fig. 11). The smaller vessels outside the cup area react only slightly or not at all. The significance of this fact is not apparent at this time. Were this reaction limited to the capillary walls, one might assume that it was related to the passage of gonadotrophin across the wall, but the walls of venules and arterioles also take the stain.

Duration of activity and fate of endometrial cups in relation to disappearance of gonadotrophin from the blood. As indicated in the section above, the cups begin to develop when the fetus has a crown-rump length of approximately 2.0 cm. Their duration of activity is indicated in Table 2. They reach their full development when the fetus has a crown-rump length of approximately 4.0 cm. and remain, as a rule, with very little change until a crown-rump length of 10.0 cm. is attained.

Desquamation of the cups usually begins when the fetal length is between 10 and 20 cm. and is completed at a crown-rump length of 30 cm. In two instances in which desquamation was underway with a fetal length of 6.0 cm., resorption of the fetus was taking place. This confirms earlier

TABLE 2. DATA ON THE DEVELOPMENT AND FATE OF THE ENDOMETRIAL CUPS AND THE ALLANTOCHORIONIC POUCHES

Crown-rump length of fetus	Total number of cases in class	Endometrial cups (Number of cases)				Allantochorionic pouches	
		Developing	Fully developed	Desquamating	Desquamated	Present	Absent
cm.							
2.0 to 2.9	4	4	None	None	None	None	4
3.0 to 5.9	11	3	8	None	None	None	11
6.0 to 10.9	23	None	19	2	2	None	23
11.0 to 14.9	12	None	5	4	3	2	10
15.0 to 20.9	14	None	6	2	6	5	9
21.0 to 29.9	32	None	1	4	27	25	7
30.0 to 39.9	9	None	None	1	8	3	6
40.0 to 81.3	4	None	None	None	4	2	2

studies on blood serum (Cole and Saunders, 1935) which indicated that resorption of the fetus may occur in the horse. At the time of desquamation, the cups are pale, opaque and less turgid. The entire structure is slowly detached. The detached cup either lies free in the space between the endometrium and the allantochorion or is enclosed in the allantochorionic pouch which we will describe later. Though there are some exceptions, the concentrations of hormone in the blood begin to decrease when the fetus attains a crown-rump length of 11.0 cm. which is the approximate time that desquamation begins (Table 3). In nearly every instance in which the hormone concentration of the blood remains high beyond the normal time, the cups likewise show evidence of prolonged secretory activity. In most instances, the blood contains less than 1 I.U. per cc. at a fetal length of 25.0 cm. This fetal length would correspond to approximately the 150th day of pregnancy.* The factor, or factors, involved in controlling the

TABLE 3. GONADOTROPHIN IN THE BLOOD OF A PORTION OF THE MARES USED IN THIS STUDY

(These data are from samples collected in 1950)

Crown-rump length of fetus	I.U. *of gonadotrophin per cc. of serum in individual mares*
cm.	
2.0 to 2.9	0.1, 3, 6.
3.0 to 5.9	11, 23, 77, 82, 82, 86, 160.
6.0 to 10.9	6, 33, 60, 65, 112, 150, 155, 160, 200, 205, 208, 250, 296.
11.0 to 14.9	<1, 2, 8, 98.
15.0 to 20.9	0.5, 1, 10, 106.
21.0 to 29.9	0.2, 0.2, 0.4, <1, <2, 2, 12, 23, 25, 59, 106, 155.
30.0 to 44.5	<0.1, <0.1, <0.2, <1.

activity of the endometrial cups remains an interesting topic for speculation. The cups begin their formation within relatively narrow time limits following conception, but their span of functional activity appears to vary greatly. How much this variation depends upon genetic constitution and how much on the environment is impossible to state at this time. Day and Rowlands (1947) showed a considerable difference in potency between Welsh and Shetland ponies. Furthermore, they observed a diminishing secretory rate in succeeding pregnancies which did not appear to be related to the age or parity of the animal, the diet, or the concentration of gonadal hormones in the blood. We, likewise, have observed marked yearly differences in the amount of gonadotrophin in the endometrial cups and secre-

* One mare sacrificed in an earlier study (Cole and Goss, 1943) on the 105th day of pregnancy had a fetus measuring 19.7 cm. Furthermore, studies on the concentration of the hormone in the blood (Cole and Saunders, 1935) showed that the hormone disappears at about the 150th day.

tion (Table 4). As the animals used in this study were collected on the range and shipped long distances for slaughter, it may well be that more severe environmental conditions were encountered in 1950 than in 1949.

TABLE 4. AVERAGE AMOUNT OF GONADOTROPHIN PER MARE IN ENDOMETRIAL CUPS PLUS ENDOMETRIAL CUP SECRETION AT VARYING STAGES OF PREGNANCY IN 1949 AND 1950

Crown-rump length of fetus	1949			1950		
	Number of cases	Average weight of cups plus secretion	Gonadotrophin (thousands of I.U.)	Number of cases	Average weight of cups plus secretion	Gonadotrophin (thousands of I.U.)
cm.		gm.			gm.	
2.0– 4.9	None	—	—	12	3.5	21
5.0– 9.9	3	10.8	252	14	8.0	56
10.0–14.9	8	5.2	172	11	5.5	73
15.0–19.9	7	7.2	761	4	10.5	150
20.0–24.9	9	2.8	98	9	8.6	133
25.0–29.9	5	1.4	79	10	5.2	42
30.0–34.9	2	1.6	16	3	2.9	14

The duration of functional activity of the endometrial cups can be estimated by taking into account two factors: the period in which the hormonal level in the blood continues to rise; the period in which the amount of hormone in the endometrial cups (plus secretion) continues to increase. As mentioned above, the concentration of hormone in the blood drops in most instances when the fetal crown-rump length exceeds 10 cm. Thus if hormone is secreted into the blood after this period, it would seem that the hormone is being destroyed or excreted faster than the secretory rate. On the other hand, the greatest amount of hormone in the endometrial cups and secretion is found at a fetal length of 15 to 20.9 cm. (Table 5). This clearly indicates that the endometrial cups are acting as exocrine glands after their endocrine function subsides. Table 5 further shows that the amount of hormone in the endometrial cup secretion increases rapidly after a fetal length of 11.0 cm. is attained.

The allantochorionic pouches. In approximately 60% of the cases studied, allantochorionic pouches filled with endometrial cup secretion form in juxtaposition with the endometrial cups. The accumulated endometrial cup secretion pushes out the allantochorion thus forming a sac which dangles in the allantoic cavity (Figs. 2, 3 and 4). As seen in Table 2, these pouches are first encountered after a fetal length of 11 cm. is reached. In those cases in which pouches are not formed, the endometrial cup secretion and the desquamated cups lie free in the space between the endometrium and chorion. The pouches may be absent even though an abundant amount of secretion is present.

The size and number of the pouches varies greatly in different mares

TABLE 5. GONADOTROPHIN IN ENDOMETRIAL CUPS AND IN ENDOMETRIAL CUP SECRETION IN PROGRESSIVE STAGES OF PREGNANCY
(These data are from samples collected in 1949)

Crown-rump length of fetus	Endometrial cups		Endometrial cup secretion	
	I.U. per mg. in individual cases	Total in individual cases (thousands of I.U.)	I.U. per mg. in individual cases	Total in individual cases (thousands of I.U.)
cm.				
5.0 to 5.9	27	349	27	2
6.0 to 10.9	20, 21, 33, 16, 0.4.	107, 296, 225, 130, 0.2.	None,† 23, 130, 41, 60.	None, 0.6, 45, 11, 3.
11.0 to 14.9	4, None,* 35, None,* 15.	13, None, 321, None, 129.	100, 11, 316, 12, 120.	89, 16, 370, 2, 132.
15.0 to 20.9	29, None,* 10, 9, None,* 11, 16, None.*	326, None, 68, 35, None, 63, 99, None.	360, 48, 200, 79, 210, 105, 246, <0.5.	1150, 138, 1400, 62, 480, 360, 1150, <0.34.
21.0 to 29.9	None in 13 cases*	None	41, 4, 15, 8, 31, 15, 3, 58, 0.7, 2, 13, 91, 0.7.	187, 8, 36, 5, 51, 48, 4, 547, 0.075, 2, 9, 385, 0.5.
30.0 to 36.4	None in 3 cases*	None	10, None,† None.*	32, None, None.

* Cups desquamated included with secretion.
† No secretion present.

They have a length ranging from 0.5 cm. to 10 cm. Their diameter at the base may exceed 1 cm. and there is a tendency for the neck of the pouch to be constricted. This constriction may completely close the neck and in this instance, the hormone in the pouch cannot be absorbed into the maternal circulation but only into the fetal circulation through the allantoic vessels. The secretion is gradually absorbed from these pouches and as this occurs they diminish in size. Though the pouches are usually small and few in number in stages beyond 30 cm. crown-rump length, they have been observed as late as 63 cm.

The secretion in the pouches found in later stages of pregnancy becomes greyish and muddy in appearance as compared to the amber colored, very sticky secretion found in newly formed pouches. The hormone content is also very low in stages beyond 30 cm.

Schauder (1912) gave an excellent account of the formation of these pouches. Less complete descriptions had been made earlier by Bonnet (1889) and Thieke (1911). Schauder referred to these structures as pendulous hippomanes. This nomenclature is in line with his view that the contents of the pouches passed through the allantochorion to form the free-floating hippomanes first described by Aristotle. We feel confident, however, that there is no connection betweeen the allantochorionic sacs and

the hippomanes as we have found these free-floating hippomanes at stages preceding the appearance of the allantochorionic pouches. Rather it, appears that, as the endometrial cup secretion in these pouches is absorbed into the fetal circulation, the pouches shrink in size and eventually are obliterated.

DISCUSSION

It is of interest to relate the various changes occurring in the reproductive organs with each other and with variations in hormone levels:

Gonadotrophin in blood. Gonadotrophin appears in the blood at approximately the 40th day of pregnancy or at a fetal crown-rump length of 2.0 cm. (Cole and Hart, 1930; Catchpole and Lyons, 1934; Cole, Howell and Hart, 1931; Cole and Saunders, 1935). The greatest concentration in the blood is found at approximately the 80th day or a fetal length of approximately 10 cm. The hormone disappears from the blood at approximately the 150th day when the fetus has a crown-rump length of 30 cm.

Endometrial cups. The cups are first observed at a crown-rump length of 2.0 cm. coincidently with the appearance of gonadotrophin in the maternal blood. Desquamation of the cups begins at 10 to 20 cm. crown-rump fetal length and is complete at 30 cm. The time of disappearance of the hormone in maternal blood corresponds roughly to the the time when desquamation of the endometrial cups is complete. As Catchpole *et al.* (1935) found residual equine gonadotrophin in the blood of a gelding 27 days after the intravenous injection of 90,000 rat units, it appears that the rate of destruction and/or excretion is slow. One would conclude, therefore, that the endocrine secretory activity of the cups ceases considerably before desquamation is complete.

Formation of multiple corpora lutea in maternal ovaries. A fresh set of corpora lutea appear in the ovaries shortly after the fetus reaches a length of 2.0 cm. coincident with the appearance of gonadotrophin in maternal blood. As many as 11 new corpora are found. Amoroso *et al.* (1948) found that ovulation occurred at the time that gonadotrophin appeared in the blood of the pregnant mare. Regression of corpora lutea begins at approximately the 150th day of pregnancy and only vestiges are seen when the fetal length exceeds 40 cm. (Cole, Howell and Hart, 1931).

Development of fetal gonads. Rapid development begins at 2.0 cm. crown-rump length with the gonads reaching a maximal prenatal development at 60 cm. (Cole *et al.*, 1933). Fetal gonads weigh approximately 60 gm. at 60 cm. crown-rump length and regress to 20 gm. at birth. Residual hormone in the allantochorionic pouches may be absorbed slowly into the fetal circulation and account for the continued accelerated development of the fetal gonads after regression of the maternal gonads occurs.

Allantochorionic pouches. Data in the present paper show that the pouches appear at a fetal length of 10 to 15 cm. The greatest accumulation of gonadotrophin in the secretion occurs at 15 to 20 cm. Hormone is present in the pouches until a crown-rump length of 30 cm. is reached.

Estrogen in urine. Estrogen appears in the urine at approximately the 70th day or at a fetal length of 9 to 10 cm. (Cole and Saunders, 1935). The hormone persists in the urine until parturition though its highest concentration is found at the 250th day. Evidence submitted by Hart and Cole (1934) indicated that the fetal placenta is the source of this hormone.

SUMMARY

One hundred and eight pregnant mares with fetuses ranging in size from 2.2 to 81.3 cm. (crown-rump length) were used in these studies. The gross appearance of the endometrial cups and allantochorionic pouches was observed in all animals. Histological study was made on sections from cups in 22 mares distributed over the period when cups are present. The gonadotrophic potency was determined for the cups and the secretion separately in 34 mares. In 64 mares, the combined gonadotrophic activity of the cups plus secretion was evaluated. In 47 cases, assays were made on the blood serum.

The cups begin to form in the endometrium at a fetal crown-rump length of 2.0 cm., reach full development at approximately 4.0 cm. and undergo little change until approximately 10.0 cm. Desquamation of the cups begins at approximately 10.0 cm. and is complete at a crown-rump length of 30.0 cm. The most marked histological changes occurring are the enlargement of the uterine glands and the hypertrophy of the stomal cells.

Gonadotrophic activity of the endometrial cups was found in the earliest stages at which the cups could be detected and reached a maximum at approximately 15.0 to 20.9 cm.

The pressure of the accumulating endometrial cup secretion pushes in the allantochorion until a sac-like structure, which is referred to as the allantochorionic pouch, is formed. These pouches are not always found even though desquamating cups and large amounts of secretion are present. The pouches diminish in size and have disappeared in most stages beyond 30 cm. crown-rump length.

Further evidence that the endometrial cups secrete gonadotrophin is presented. The hormone appears in the blood coincident with the initial development of the cup. When stained histochemically for glycoprotein, the endometrial cup secretion, the uterine epithelium and the glandular epithelium are stained while the large "decidual" cells give little evidence of any reaction, indicating that the epithelial cells in the cup area are the source of gonadotrophin.

REFERENCES

AMOROSO, E. C., J. H. HANCOCK AND I. W. ROWLANDS: *Nature* **161:** 355. 1948.

BONNET, R.: *Anat. Anz.* **4:** 17. 1889.

CATCHPOLE, H. R. AND W. R. LYONS: *Am. J. Anat.* **55:** 167. 1934.

CATCHPOLE, H. R., H. H. COLE AND P. B. PEARSON: *Am. J. Physiol.* **112:** 21. 1935.

COLE, H. H. AND G. H. HART: *Am. J. Physiol.* **93:** 57. 1940.

COLE, H. H., C. E. HOWELL AND G. H. HART: *Anat. Rec.* **49:** 199. 1931.

COLE, H. H., G. H. HART, W. R. LYONS AND H. R. CATCHPOLE: *Anat. Rec.* **56:** 275. 1933.

COLE, H. H. AND F. J. SAUNDERS: *Endocrinology* **19:** 199. 1935.

COLE, H. H. AND J. ERWAY: *Endocrinology* **29:** 514. 1941.

COLE, H. H. AND H. GOSS: In *Essays in Biology in honor of Herbert M. Evans.* Univ. of Calif. Press. p. 107. 1943.

DAY, F. T. AND I. W. ROWLANDS: *J. Endocrinol.* **5:** 1. 1947.

HART, G. H. AND H. H. COLE: *Am. J. Physiol.* **109:** 320. 1934.

MCMANUS, J. F. A.: *Nature* **158:** 202. 1946.

MOSSMAN, H. W.: *Carnegie Inst. of Wash., Contributions to Embryology* **26:** 129. 1937.

SCHAUDER, W.: *Arch. f. Anat. u. Physiol. Jahrb.* **1912:** 259. 1912.

THIEKE, A.: *Anat. Anz.* **38:** 465. 1911.

EFFECT OF A PITUITARY GONADOTROPHIN ON THE OVARIES OF HYPOTHYROID RATS

RALPH G. JANES

The Departments of Anatomy, State University of Iowa, College of Medicine, Iowa City, Iowa, and St. Thomas's Hospital Medical School, London, S.E. 1, England

IT HAS been shown by several investigators that thyroidectomy or the administration of goitrogenic substances is followed by changes in the ovary. Williams, Weinglass, Bissel and Peters (1944) found that ovarian weight decreased after thiouracil administration; Kòpf, Loeser and Meyer (1948) and Szontágh and Lichner (1951) observed that an experimentally induced hypothyroidism resulted in a decrease in the number of follicles and marked luteinization. Janes (1944) and Janes and Bradbury (1952) reported atrophy of the ovary in some rats which have been thyroidectomized or given thiourea for a period of 9 months.

The observations suggest that, in the absence of normal thyroid function, the ovaries react abnormally to the circulating gonadotrophins. Furthermore, when gonadotrophins were administered for short periods of time to rats that were thyroidectomized or given thiouracil, the ovarian response was increased in some instances, decreased in others (Smelser and Levin 1941; Bischoff and Clark 1941a and b; Johnson and Meites 1950).

In view of the importance of a better understanding of the effect of gonadotrophins in hypothyroid animals, the present experiments are concerned with the effects of varying doses of a gonadotrophin on animals which were given a goitrogen or thyroidectomized for different periods of time.

METHODS

Two hundred and twenty-four female rats of the Long-Evans strain, weighing 85-95 gm. at the beginning of the experiment, were used. Of these, one group was fed on 0.5% propylthiouracil[1] in Rockland rat ration for periods varying from 14–70 days; another group was thyroidectomized for 40 or 70 days; while a third group was used as controls. All received subcutaneous injections of gonadotrophin[2] twice daily during the

Received for publication October 6, 1953.

[1] Supplied through the courtesy of Lederle Laboratories, Pearl River, New York.

[2] The gonadotrophin (*Synergist*) was supplied through the courtesy of Parke-Davis & Co., Detroit, Michigan. It was a 40% acid-alcohol extract of beef and hog pituitaries, which was further fractionated with ammonium sulphate.

later stages of the experimental period, the dosage and duration of treatment with this being varied in different groups.

At the end of the experimental periods the pituitaries, adrenals, thyroids and ovaries were weighed and sectioned for histological examination. In the thyroidectomized animals, the connective tissues on the surfaces of the larynx and upper trachea were removed, sectioned and examined for thyroid remnants. Any animals showing such remnants, in all about 50%, were discarded from the series.

In addition, two series of uninjected controls were similarly examined, the one averaging 86 gm. in body weight and thus approximating to the average weight of experimental animals at the beginning of the experiment, and the other averaging 116 gm. body weight.

RESULTS

These are summarized in Tables 1 and 2. In pilot experiments normal animals receiving large dosage of gonadotrophin (11 A.U.[3] twice daily), for as short a period as 3 days, showed a trebling of the ovarian weight as compared with the uninjected controls. A slightly smaller dose (9 A.U. twice daily) of gonadotrophin continued for a longer period (10 days) gave an even more marked response, while the same dose continued for 20 days resulted in a terminal diminution in ovarian weight as compared with the rats in the 10 day experiment (Table 1). In consequence a much smaller dose, $4\frac{1}{2}$ A.U. twice daily, was used in the remaining experiments and continued over a period of 10 days in one group and 20 in another. During these periods the ovarian response was maintained when judged by the weight of these organs. There was a slightly increased response in the 20 day group as compared with the 10 day group.

In animals treated with thiouracil over a period of 14 days, 22 A.U. of gonadotrophin daily during the last three days of the experiment evoked a significantly less ovarian response when compared with the corresponding controls; this diminution was more marked in animals which had received thiouracil for a longer period (28 days). A similar response was seen in thiouracil treated animals receiving 18 A.U. of gonadotrophin daily for a period of 10 days, but there was an ovarian response much greater than seen in the corresponding controls when the gonadotrophin was continued at this level for 20 days (Table 1). Again, using $4\frac{1}{2}$ A.U. twice daily in rats which had received thiouracil for as long as 40 days there was less gain in ovarian weight after 10 days, but a considerable increase after 20 days treatment (Table 2).

In rats which had received thiouracil for 70 consecutive days, the injec-

[3] The A.U. (Activation Unit) is the amount of pituitary gonadotrophin which whem combined with 12.5 international units of chorionic gonadotrophin would give with 6 injections 30 mg. ovaries in 72 hours (chorionic alone gave 15 mg. ovaries). There were 37 A.U. per mg. of extract and each mg. contained 4–6 rat units of gonadotrophin.

TABLE 1. EFFECT OF GONADOTROPHIN ON OVARIES OF NORMAL AND THIOURACIL TREATED RATS

Treatment	Amount of gonadotrophin per day	Days treated with gonadotrophin	No. of rats	Body wt.-gm.	Ovaries wt. mg./100 gm.	% ovarian cysts
Control	None		8	116	24.2 ± 1.5*	0
Control	22 A.U.	3	6	110	78.7 ± 6.2	17
Thiouracil 14 days	22 A.U.	3	6	109	59.5 ± 3.8	17
Thiouracil 28 days	22 A.U.	3	10	107	43.2 ± 9.0	20
Control	18 A.U.	10	8	133	116.8 ± 18.7	0
Thiouracil 40 days	18 A.U.	10	8	118	63.0 ± 2.5	38
Control	18 A.U.	20	8	144	93.4 ± 15.0	50
Thiouracil 70 days	18 A.U.	20	8	107	291.5 ± 45.0	25

* Standard Error of mean $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

jection of 9 A.U. of gonadotrophin resulted in considerable increase in ovarian weight both in the 10 and 20 day groups as compared with the controls which had not received thiouracil (Table 2). It would thus appear either that the duration of thiouracil treatment (particularly if thiouracil was fed for 40 or more days) directly modified the ovarian response to gonadotrophin or that, during the period of thiouracil treatment, there was an accumulation of gonadotrophin in the blood and the ovarian response varied for some unknown reason according to the concentration of this substance in the body fluids. In view of the varying response to the differing levels of gonadotrophin in the controls the latter seemed more likely.

This was borne out by experiments on thyroidectomized animals. In two groups thyroidectomized 40 and 70 days the ovarian response to 9 A.U. of gonadotrophin daily for 10 or 20 days was a considerable increase in weight, more marked than in any of the corresponding thiouracil treated animals.

It is difficult to compare the present observations with results obtained by other workers because the latter had given gonadotrophin for only 3 or 4 days and the length of time the animals had been subjected to a goitrogen or thyroidectomy was in most cases, less than in the present studies. Smelser and Levin (1941) have stated that if 21 day old rats were thyroidectomized for 2 or 3 days and given a gonadotrophin for 3 days,

TABLE 2. EFFECT OF GONADOTROPHIN ON HYPOTHYROID RATS

Treatment	Amount of gonadotrophin per day	Days treated with gonadotrophin	No. of rats	Body wt. gm.	Ovaries wt. mg./100 gm.	Ovarian cysts % of rats	Thyroids wt. mg./100 gm.	Pituitary wt. mg./100 gm.	Adrenals wt. mg./100 gm.
Control	None		11	86	24.6± 1.7		8.0	5.4	25.0
Control	None		8	116	24.2± 1.5		9.2	3.9	24.2
Control	9 A.U.	10	17	130	68.0±10.5	29	7.8	5.1	25.7
Control	9 A.U.	20	17	148	72.4± 9.4	41	10.1	5.3	17.0
Thiouracil 40 days	None		8	120	22.0± 3.1		62.3	5.8	19.8
Thiouracil 40 days	9 A.U.	10	9	93	52.8± 6.5		67.3	7.3	25.0
Thiouracil 40 days	9 A.U.	20	8	115	165.6±31.0	88	86.6	8.0	23.3
Thiouracil 70 days	None		11	102	22.4± 1.8	9	96.6	7.2	22.2
Thiouracil 70 days	9 A.U.	10	10	110	127.1±11.7	20	94.0	9.1	22.3
Thiouracil 70 days	9 A.U.	20	10	98	278.0±37.2	80	102.5	9.4	23.4
Thyroidect. 40 days	None		8	143	28.2± 2.2			6.8	19.5
Thyroidect. 40 days	9 A.U.	10	8	130	107.4±18.0	100		8.2	19.5
Thyroidect. 40 days	9 A.U.	20	8	142	243.5±43.0	100		8.8	15.1
Thyroidect. 70 days	None		9	145	31.7± 2.8			8.2	22.9
Thyroidect. 70 days	9 A.U.	10	13	143	114.4±15.0	85		7.6	16.9
Thyroidect. 70 days	9 A.U.	20	13	146	224.0±33.8	92		9.0	15.7

there was a greater ovarian response than in normal test animals. However, this does not appear to be clear from the data presented. Bischoff and Clarke (1941a) reported that if 21 day old rats were thyroidectomized for a period of 42 days and given a pituitary gonadotrophin for 4 days there was an increased ovarian response. On the other hand, if the animals were thyroidectomized for only 3 days and given pregnant mare's serum (PMS) the next three days, there was no difference in ovarian response provided the dosage was proportional to the body weight (Bischoff and Clarke 1941b). More recently, Johnson and Meites (1950) reported that when PMS was given for 4 days to rats which had received thiouracil for up to 15 days, the ovarian response was greater than in controls similarly treated with PMS. However, if the thiouracil was continued for 20 days the ovaries of the PMS treated rats were smaller than found in the controls.

It is, however, clear from the present work that the ovarian response to gonadotrophin not only varies with the dosage but also the duration of the treatment. Furthermore the response is modified considerably by prior thiouracil treatment or thyroidectomy. If of short duration, thiouracil treatment diminishes the ovarian response to small doses of gonadotrophin while if of long duration, the response to gonadotrophin is exaggerated as compared with that in control animals both with large and small doses of gonadotrophin. In this latter group, though the response is of a similar

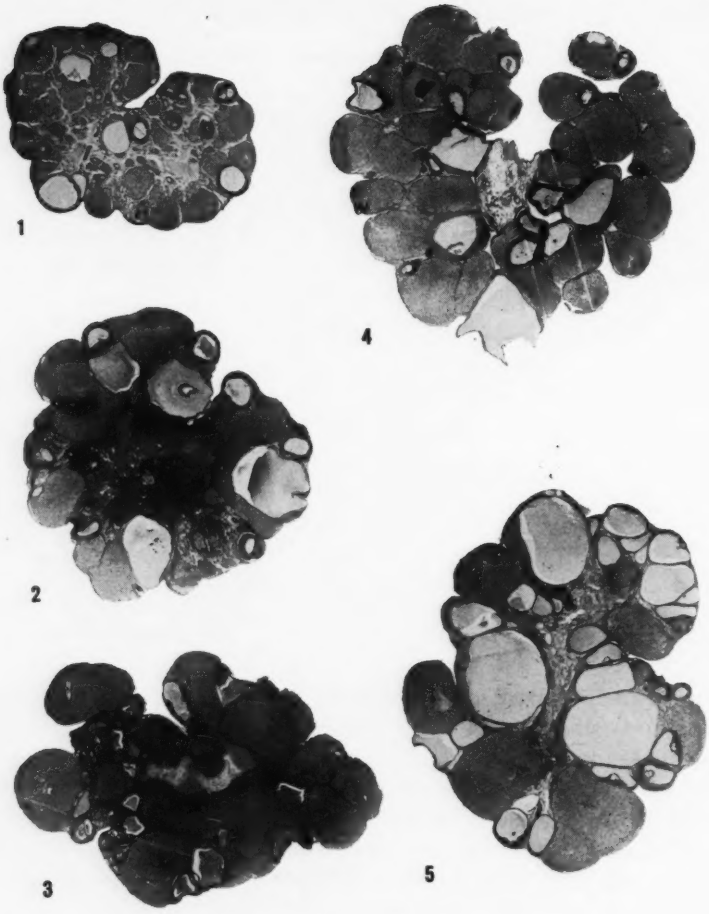


PLATE I

EXPLANATION OF FIGURES

All rats given daily injections of 9 A.U. of *Synergist* for 20 days. Magnification ×6.5 throughout.

FIG. 1. Ovary from normal rat. 37 mg./kg. body weight. Small corpora lutae, large atretic follicles and small normal follicles are present.

FIG. 2. Ovary from rat given propylthiouracil for 40 days. 147 mg./kg. body weight. Large corpora lutea, atretic follicles and cystic follicles in which theca interna is luteinized may be seen. Very few small follicles are present.

FIG. 3. Ovary from rat given propylthiouracil for 70 days. 286 mg./kg. body weight. This ovary consists chiefly of large corpora lutea and some atretic follicles.

FIG. 4. Ovary from rat thyroidectomized for 40 days. 332 mg./kg. body weight. Ovary consists chiefly of large corpora lutea, some cysts and atretic follicles.

FIG. 5. Ovary from rat thyroidectomized for 70 days. 141 mg./kg. body weight. Some large corpora lutea, large cysts and atretic follicles are present. There are no normal small follicles.

nature, the magnitude of the response is greater in certain instances after thyroidectomy than after the administration of thiouracil. While hypothyroidism may lead to an accumulation of gonadotrophin in the blood which in its turn modifies the response to injected gonadotrophin by augmenting the total effect, direct action of hypothyroidism on the target organ cannot be eliminated, and indeed seems likely.

Unfortunately ovarian weight changes do not afford an accurate criterion for measuring gonadotrophin stimulation. An increase in weight might result from (a) the production and growth of new follicles. (b) a massive luteinization of existing follicles, or (c) the formation of ovarian cysts. When the ovaries which showed the increase in weight were examined microscopically it was found that the weight increase was caused chiefly by the large corpora lutea and to some extent by ovarian cysts, and thus factors (b) and (c) were involved.

The ovaries from control rats and from animals that were hypothyroid for periods up to 70 days had similar weights and microscopic structure. The hypothyroid condition prevented the normal growth pattern and the ovaries from these animals remained immature. The microscopic appearance of ovaries from representative groups of hypothyroid rats treated with gonadotrophin for 20 days is shown on Plate I. In general, animals showing a diminished ovarian response had ovaries similar to the one shown in Figure 1. Small normal and large atretic follicles were present and the corpora lutea remained rather small. On the other hand, animals showing an increased response, compared with their controls, had many large corpora lutea, atretic follicles and in most cases some cystic follicles (Fig. 2–5). The theca interna surrounding the cysts was generally luteinized. A few normal follicles were usually found in ovaries of animals that were hypothyroid for 40 days but were absent in rats thyroidectomized for 70 days (Fig. 5). The increased ovarian weight response as seen in the hypothyroid animals appears to be a qualitative rather than a quantitative effect and results from the luteinizing rather than a follicular stimulating factor.

In addition to the ovary, certain other organs were weighed in an effort to evaluate the degree of hypothyroidism and to determine, if possible, whether the gonadotrophin, *per se*, was causing gross changes in these organs. Although the amount of thiouracil given to the rats was somewhat large, the weights of the thyroids were similar to those ordinarily found following the administration of this goitrogen (Table 2).

The adrenals and pituitaries showed the usual alterations in weight as seen in hypothyroid rats (Janes, 1946). The pituitaries were enlarged in animals receiving thiouracil as well as in those that were thyroidectomized. Gonadotrophin apparently did not alter their weights. In general the adre-

nals were smaller in the hypothyroid rats, and this was particularly noticeable in the thyroidectomized animals (Table 2).

SUMMARY

Immature rats either were fed propylthiouracil or were thyroidectomized for periods up to 70 days. A gonadotrophin was given at the end of each experimental period.

The ovarian response to gonadotrophin varies with the dosage and length of treatment and is modified by prior thiouracil treatment or thyroidectomy. If of short duration thiouracil reduces the gonadotrophic effect while if of longer duration the response to gonadotrophin is much greater than found in control animals. Thyroidectomy results in an increased response to gonadotrophin and the ovarian weights in some instances are even greater than in the thiouracil treated. The increased ovarian response to gonadotrophin is a qualitative rather than a quantitative effect and is apparently caused by a luteinizing rather than a follicular stimulating factor.

REFERENCES

BISCHOFF, F. AND G. J. CLARKE: *Endocrinology* **29**: 27. 1941a.
BISCHOFF, F. AND G. J. CLARKE: *Endocrinology* **29**: 162. 1941b.
JANES, R. G.: *Anat. Rec.* **90**: 93. 1944.
JANES, R. G.: *Am. J. Physiol.* **145**: 411. 1946.
JANES, R. G. AND J. T. BRADBURY: *Proc. Soc. Exp. Biol. & Med.* **79**: 187. 1952.
JOHNSON, T. N. AND J. MEITES: *Proc. Soc. Exp. Biol. & Med.* **75**: 155. 1950.
KOPF, R., A. LOESER AND G. MEYER: *Klin. Wschr.* **26**: 202. 1948.
SMELSER, G. K. AND L. LEVIN: *Endocrinology* **28**: 233. 1941.
SZONTÁGH, F. E. AND G. LICHNER: *Experientia* **7**: 384. 1951.
WILLIAMS, R. H., A. R. WEINGLASS, G. W. BISSEL AND J. B. PETERS: *Endocrinology* **34**: 317. 1944.

NOTES AND COMMENTS

POLY-ESTRADIOL PHOSPHATE (PEP); A LONG-ACTING WATER SOLUBLE ESTROGEN

It has been reported previously that some phosphoric acid esters of estradiol-17β,[1] especially estradiol 3:17-diphosphate inhibit kidney alkaline phosphatase (Aldman, Diczfalusy and Rosenberg, 1948; Aldman, Diczfalusy, Högberg and Rosenberg, 1951). Subsequent work, however, showed that the enzyme-inhibiting property is not confined to estradiol phosphates alone and that polymeric phosphates of phloretin and other aromatic polyphenols are also capable of inhibiting alkaline phosphatase (Diczfalusy, Fernö, Fex, Högberg, Linderot and Rosenberg, 1953 a). This last mentioned observation suggested the possibility that the previously reported inhibition of the enzyme by estradiol diphosphate might result from the presence of some contaminating polymeric estradiol phosphate. Accordingly poly- estradiol phosphate (hereafter PEP) was synthesized following the technique for the synthesis of polymeric phosphates of aromatic polyphenols, as described in a previous paper (Diczfalusy *et al.*, 1953 a).[2]

Thus the investigation hereby reported was started with the aim of elucidating the connection between the inhibition of alkaline phosphatase by estradiol diphosphate on the one hand and that produced by PEP on the other.

When, however, these two substances were tested for estrogenic activity in spayed mice, the incidental observation was made that PEP, although only weakly active when assayed in the conventional Allen-Doisy test, prolonged the duration of vaginal cornification in spayed mice to an extent very much greater than that produced by the same dosage of estradiol-3-monobenzoate or ethinyl estradiol.

This paper deals with the prolonged estrogenic activity of PEP. The chemical properties of the compound and its action upon various enzymes will be reported in a subsequent paper.

EXPERIMENTAL

Preparation of PEP

This will be reported in detail by Diczfalusy, Fernö, Fex, Högberg, Linderot and Rosenberg (1953 b) elsewhere. In essence the method consists of a condensation polymerisation reaction of estradiol-17β with phosphorus oxychloride in dry pyridine at $-10°$ C. When the mixture becomes viscous, if it is hydrolyzed by the addition of crushed ice and the product formed is precipitated by dilute HCl. A white or pale yellow product results, which can be dissolved in water at slightly alkaline pH (7.5–8.0). It is dried over P_2O_5 and ground to a fine powder. This product is very resistant towards acid- or alkali hydrolysis. It was found to contain approximately 4 per cent free estradiol (measured following partition between ether and an aqueous solution of pH 8.0 by fluorimetric analysis, as described in a previous paper [Diczfalusy, 1953]), 0.012 per cent inorganic phosphorus and 7.5 per cent total phosphorus (measured by the method of Martin and Doty, 1949). The compound is assumed to be a mixture of high molecular weight polymeric estradiol phosphates of various size.

Received for publication October 8, 1953.

[1] The nomenclature used in this paper will conform with that suggested by Fieser and Fieser (1949).

[2] I am indebted to AB. Leo, Hälsingborg for the supply of this compound.

Animals

Spayed adult albino mice were used. In the classical Allen-Doisy tests the procedure described by Emmens (1939) was followed, double injections (0.1 ml.) being given in aqueous media. In the experiments where the duration of effectiveness was investigated, no priming injections were given. Smears were taken daily at 10 A.M. and were interpreted as suggested by Emmens. The effectiveness of a long-acting estrogen was measured by the duration (in days) of positive smears.

RESULTS

When assayed in the typical Allen-Doisy test, PEP administered in water, was found to be a relatively weak estrogen, exhibiting less than 10% of the potency of estradiol in water. However, when the prolonged effect of 20 μg. of PEP in propylene glycol was compared with that of the same amount of estradiol benzoate or ethinyl estradiol, the results shown in Figure 1 were obtained.

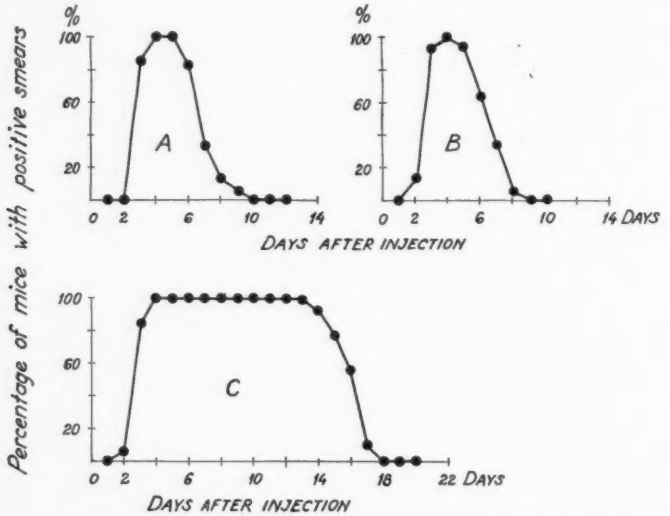


FIG. 1. Duration of vaginal cornification in spayed mice treated with a single subcutaneous injection of various estradiol-derivatives. Each point represents 24 animals. A = 20 μg. Estradiol benzoate. B = 20 μg. Ethinyl estradiol. C = 20 μg. Poly-estradiol phosphate (crude preparation). Solvent: propylene glycol. Volume: 0.1 ml.

The average duration of positive smears (±standard error) was calculated to be 4.0 ± 0.21 days in the group treated with 20 μg. of ethinyl estradiol and 4.3 ± 0.18 days in the group treated with 20 μg. of estradiol benzoate. A similar calculation gave a mean duration of 13.4 ± 0.27 days for the group treated with 20 μg. of PEP.

Since it was suspected that the prolonged estrogenic activity of PEP may depend mainly on the molecular size, possibly being associated with the high molecular weight fraction, an attempt was made to separate this fraction by dialysis. 300 mg. of PEP were dissolved in 30 ml. distilled water with the help of a few drops of N NaOH and the solution was dialyzed in a cellophane sack against 20 l. distilled water stirred continuously at 2° C. for 48 hours. The non dialyzable fraction was then precipitated by the

addition of dilute HCl, washed with ice-cold distilled water and dried at room temperature over P_2O_5. When the duration of effectiveness of 20 μg. of this preparation was compared with the same amount of the non dialyzed, crude preparation, the results shown in Figure 2 were obtained

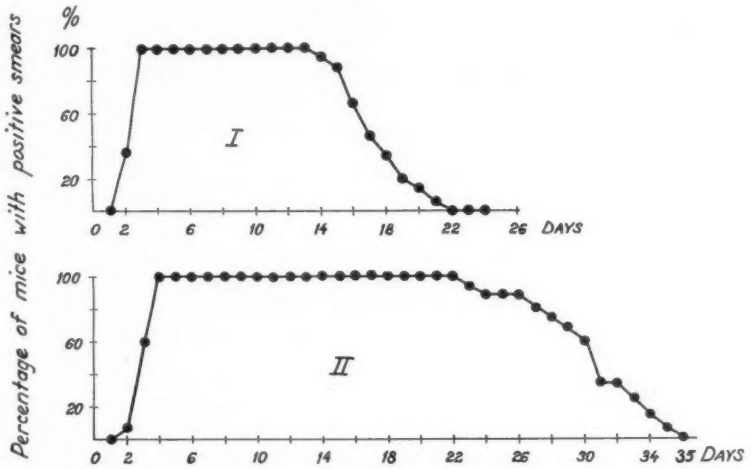


FIG. 2. Duration of vaginal cornification in spayed mice treated with a single subcutaneous injection of two poly-estradiol phosphate preparations. Each point represents 22 animals. Solvent: water. I.: 20 μg. poly-estradiol phosphate (non dialyzed, crude preparation). II.: 20 μg. of dialyzed poly-estradiol phosphate (High molecular weight fraction).

Figure 2 shows that the duration of effectiveness of 20 μg. dialyzed PEP (26.2 ± 0.99 days) greatly exceeds that of the same amount of non dialyzed crude preparation (14.5 ± 0.36 days). It would thus appear that dialysis greatly increases the duration of effectiveness of PEP due to the removal of less effective lower molecular weight material as well as impurities.

When a portion of the dialyzable fraction, which presumably contains also some inorganic phosphorus, chloride, free estradiol and some other impurities, was evaporated in an atmosphere of nitrogen and at a reduced pressure, the dry residue showed no prolonged estrogenic activity: a dose of 15 μg. given to 18 animals exhibited a duration of effectiveness of only 2.2 ± 0.31 days.

In this connection it is also of considerable interest to note that when estradiol 3:17-diphosphate[3] was administered to a group of 21 mice in a single dose of 200 μg. in aqueous solution, it did not elicit any prolonged effect. This is shown in Figure 3, where the action of 200 μg. of this compound is compared with that of 2 μg. of high molecular weight PEP.

The duration of effectiveness of 200 μg. of estradiol diphosphate is calculated to be

[3] This compound was obtained through the courtesy of Ing. B. Högberg, AB. Leo, Hälsingborg.

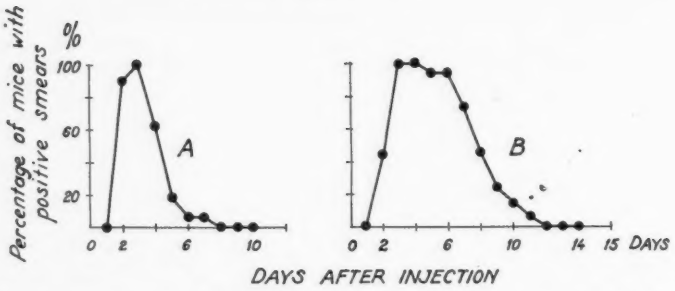


FIG. 3. Duration of vaginal cornification in spayed mice treated with 200 μg. of estradiol-3:17-diphosphate (A) and with 2 μg. of dialyzed poly-estradiol phosphate (B). Each point represents 21 mice.

2.9 ±0.22 days, whereas that of 2 μg. of high molecular weight PEP is 6.6 ±0.46 days. The difference is highly significant ($p < 0.001$). The absence of any prolonged effect following the administration of as much as 200 μg. of estradiol diphosphate may be interpreted as indirect evidence against the assumption that the previously demonstrated inhibition of kidney alkaline phosphatase by this compound would depend on the presence of contaminating polymeric high molecular weight estradiol phosphates.[4] However, a definite answer to this question must await more detailed studies.

It is also of interest to note that PEP administered by the peroral route seems to be estrogenically inactive; when a dose of 40 μg. of PEP was given by stomach tube to a group of 24 mice, in none of the animals was a positive estrous smear obtained. On the other hand, when 20 μg. of PEP (high molecular weight fraction) was administered intravenously to 20 mice, the mean duration of positive smears was 17.4 ±0.39 days. Thus it would appear that the prolonged estrogenic activity of PEP cannot entirely depend on a classical "depot"-effect.

Finally, the prolonged estrogenic activity of PEP is not influenced by precipitation of this compound by basic proteins, e.g. protamine sulfate. Such substances have proved to counteract the inhibition of kidney alkaline phosphatase or prostate acid phosphatase by PEP (Beling and Diczfalusy, unpublished observations). When a group of 25 mice were injected subcutaneously with a combination of 20 μg. PEP and 1.0 mg. of protamine sulfate, the mean duration of positive smears was 14.3 ±0.31 days. This value agrees well with the duration of effectiveness of the same amount of PEP in water (14.5 ±0.36 days) or in propylene glycol (13.4 ±0.27 days).

DISCUSSION

Possible mechanism of action of PEP. Generally speaking, the prolonged duration of effectiveness of a steroid hormone may depend among other factors on 1) delayed absorption, 2) delayed inactivation and 3) delayed excretion.

[4] Of course there is no *a priori* reason to assume that only the non dialyzable fraction inhibits the enzyme and that dialyzable polymeric estradiol phosphates are devoid of inhibitory activity. Preliminary experiments on human prostate acid phosphatase (Beling and Diczfalusy) seem to indicate, however, that the non-dialyzable fraction is a much stronger inhibitor than the crude preparation.

1) The delayed absorption of a hormone preparation ("depot"-effect) is generally ascribed to factors such as

a) the nature and volume of solvent; thus estrogens in oil have a more prolonged activity than in aqueous solutions

b) the physical state of the hormone; suspensions, microcrystals or pellets have a longer duration of effectiveness than solutions

c) introduction of certain chemical radicals; esterification generally increases the duration of effectiveness

d) "inflammatory fixation" (Burrows, 1949); the inflammatory reaction is said to inhibit the absorption of the hormone from inflamed tissues.

Although—with the possible exception of c)—these factors do not seem to influence the duration of effectiveness of PEP, it is still possible that the prolonged estrogenic activity of this substance depends on a "depot"-effect. Thus it is possible that

i) PEP is stored at the site of injection due to its ability to combine with tissue proteins, or

ii) PEP combines with serum proteins, imitating "protein-bound" estrogens and providing thus a suitable transport form of PEP in the organism. It is not unlikely that such a combination may also protect the hormone from rapid inactivation. Furthermore

iii) PEP may also be stored in certain organs, or cell-systems, *e.g.* the reticuloendothelial system.

2) Delayed inactivation should also be considered. According to current concepts it is generally assumed that estrogens are rapidly "inactivated" in the body, *i.e.* they are transformed by enzymatic processes into metabolites with diminished or no hormonal activity. PEP was found to inhibit a variety of enzymes, such as alkaline and acid phosphatase (Beling and Diczfalusy), hyaluronidase and urease (Högberg). It is therefore theoretically possible that PEP interferes with the action of some enzyme system(s) concerned with the inactivation of estrogens.

3) Delayed excretion of PEP should also be considered, since it is probable that not only the molecular weight but also the shape of the molecule is of importance in determining whether or not a compound can pass the renal filter.

In any case, as yet there is no experimental demonstration of the mechanism whereby PEP elicits a much more prolonged vaginal cornification than other estradiol derivatives. In order to throw some light on its mechanism of action, experiments are now in progress with PEP tagged with radioactive phosphorus. They will be reported in a forthcoming paper.

Standardisation of long-acting estrogens. While in the past interest was centered upon the estimation of the *intensity* of effect induced by various steroids, relatively little attention seems to have been paid to another important feature of sex hormone action, namely the *duration of effectiveness*. The importance of the latter was, however emphasized by Miescher, Fischer and Tschopp (1937), who demonstrated that certain enol esters of testosterone, although less potent than testosterone propionate in increasing the weight of the accessory reproductive organs of castrated rats, showed a much longer duration of effectiveness than the same dosage of testosterone propionate. Similarly PEP is only weakly active in the typical Allen-Doisy test, but it acts for a much longer period than any of the natural estrogens investigated so far. Thus it would appear that the intensity of hormone effect and the duration of effectiveness of the same hormone are not necessarily correlated. Therefore a bioassay method for comparing the duration of effectiveness of various long-acting preparations would be of considerable value.

Preliminary experiments, in which estradiol benzoate in propylene glycol was used

as a standard preparation, seem to indicate that statistically valid bioassays of the duration of effectiveness can be obtained in six-point, or four-point assays, by equal spacing of log doses on the standard and test preparations and using the duration time (in days) as a dependent variant. 20 animals are used on each dose level. Further experiments are needed, however, to decide the most suitable transformation of the duration time, *i.e.* whether the reciprocal or the logarithm of the time should be used as a response metameter. These experiments are now in progress.

Probable value of PEP in clinical practice. Since the amount of hormone contained in a steroid producing organ is usually small compared with that to be administered in order to produce a response in the test animal, it is generally believed that there is a continuous release of the hormone by the organ with but little storage. If this is true, the best replacement therapy would appear to be the imitation of this very condition. Since PEP is only weakly active in the typical Allen-Doisy test, whereas its duration of effectiveness greatly surpasses that of the other estrogens tested, it seems reasonable to assume that there is a slow, but continuous release of small amounts of estradiol-17β from the PEP-molecule; thus in case of a typical Allen-Doisy test, where relatively small amounts of PEP are administered, probably only subthreshold amounts of estradiol-17β are liberated and therefore a positive reaction can only be elicited in a very limited number of animals.

The the data presented in this paper would seem to justify a clinical trial of PEP.

SUMMARY

The biological properties of poly-estradiol phosphate (PEP), a water soluble high molecular weight polyester of phosphoric acid and estradiol-17β have been investigated.

The duration of the estrogenic effect produced by poly-estradiol phosphate was found to be much longer than that of estradiol benzoate or ethinyl estradiol as measured by the mouse vaginal smear technique.

It is suggested that high molecular weight polymers prepared from other steroids may also prove to have a prolonged biological activity.

Acknowledgments

I am indebted to Miss Leila Nilsson for the excellent technical assistance she has given me in the experiments.

Part of the expenses of this investigation was defrayed by a research grant from AB. Leo, Hälsingborg, Sweden.

EGON DICZFALUSY

Department of Women's Diseases
Karolinska sjukhuset
Stockholm, Sweden

REFERENCES

ALDMAN, B., E. DICZFALUSY AND T. ROSENBERG: *Acta Chem. Scand.* **2**: 529. 1948.

ALDMAN, B., E. DICZFALUSY, B. HÖGBERG AND T. ROSENBERG: *Biochem. J.* **49**: 218. 1951.

BELING, K. G. AND E. DICZFALUSY: Unpublished data.

BURROWS, H.: Biological action of sex hormones. Cambridge. p. 150, 1949.

DICZFALUSY, E.: *Acta endocrinol.* **12**: Suppl. XII. 1953.

DICZFALUSY, E., O. FERNÖ, H. FEX, B. HÖGBERG, T. LINDEROT AND T. ROSENBERG: *Acta Chem. Scand.* **7**: 913. 1953a.

DICZFALUSY, E., O. FERNÖ, H. FEX, B. HÖGBERG, T. LINDEROT AND T. ROSENBERG: in preparation, 1953b.

EMMENS, C. W.: Med. Research Council (Brit.) *Special Rept. Ser.* No. 234. H.M. Staff Office, London. 1939.

FIESER, L. F. AND M. FIESER: Natural products related to phenanthrene. 3rd Ed. (Reinhild, N.Y.) 1949.

HÖGBERG, B.: Personal communication

MARTIN, J. B. AND D. M. DOTY: *Anal. Chem.* **21**: 965. 1949.

MIESCHER, K., W. H. FISCHER AND E. TSCHOPP: *Nature* **140**: 726. 1937.

EVIDENCE AGAINST DESTRUCTION OF EOSINOPHILS BY GLUCOCORTICOIDS AS SHOWN BY *IN VITRO* EXPERIMENTS

Several explanations for the eosinopenia induced by ACTH and cortisone, recently reviewed by Essellier and Wagner (1952a), have been advanced. Godlowski (1952, 1953) and Muehrcke (1952) suggested a direct destruction of the eosinophils *in vivo* by adrenocortical hormones on the basis of their findings of an eosinolytic effect *in vitro* of compounds E and F. Padawer and Gordon (1952) arrived at the same conclusions after having observed an increased number of degenerating eosinophilic forms in the peripheral blood, following subcutaneous injection of cortisone. Baldridge (1951) and Coste (1952) however, negate any direct eosinolytic effect of these hormones *in vitro* and Panzenhagen and Speirs (1953) report no changes in eosinophils in peritoneal fluid of mice after intraperitoneal injection of cortisone. Other findings also do not lend support to the concept that the eosinopenic state may result from actual destruction of eosinophils by adrenocortical hormones. Essellier and Wagner (1952b) for instance, observed that the blood eosinopenia following ACTH injection is not accompanied by any modifications of the medullary eosinophils; the same is reported by Quittner *et al.* (1951) in cortisone induced eosinopenia. The inhibition of the eosinopenic effect of ACTH and cortisone after blockade of the reticulo-endothelial system by vital dyes, as described by Essellier and Wagner (1952a) brings additional evidence against direct effects of cortical hormones on the eosinophils.

The work reported here, based on a large quantity of material and on incubation experiments of long duration (24 hours), was conducted with the purpose of obtaining a better insight in this controversial question.

METHODS

Preliminary experiments: Before performing experiments with steroids it was necessary to obtain information on the change in the total leukocyte and eosinophil counts of blood samples made uncoagulable and incubated for 24 hours. For this purpose, blood samples from six human subjects with eosinophil counts ranging from 366 to 8100 cells per cu. mm. of blood were tested. Three blood samples of 5 cc. were obtained by venipuncture from each subject: one was difibrinated by careful shaking with glass beads, the second citrated (5 cc. blood+1 cc. citrate 3.5%) and the third heparinized (1 mg. heparin in 5 cc. blood). All samples were incubated at 37° C. in test tubes 1 cm. in diameter. Total leukocyte counts as well as eosinophil counts by Randolph's phloxine method (1949) were performed in duplicate, immediately after withdrawing blood from the patient and after 2, 4, 6 and 24 hours. Smears stained by the May-Grünwald technic were obtained after the blood sampling and after 6 and 24 hours incubation. The result of these pilot experiments are included in the statistical analysis of the control experiments without steroids.

Steroid experiments and controls: Two mg. of various steroids having a known eosinopenic effect *in vivo* were added to defibrinated or citrated blood samples and incubated for

Received for publication November 2, 1953.

24 hours. A control with the same blood but without steroid addition was run in parallel with each steroid test. Tests were carried out on blood samples from 30 subjects with eosinophil counts ranging from 168 to 17,995 cells per cu. mm. The steroids used were *Cortone* and *Hydrocortone* (Merck) Cortisone and compound F (Ciba) and the more soluble preparations, cortisone aldehyde (53R693 Merck) and dehydrocortisone acetate (52R6000 Merck).[1]

RESULTS

Controls: It is seen from Table 1 that the mean total leukocyte and eosinophil counts in control and pilot experiments, *i.e.* in blood samples incubated without steroids, never undergo a statistically significant drop even after 24 hours incubation, despite some variations in the number of cells at the times of the various counts.

Steroid experiments: The computation of the standard errors of the mean values for the counts on each variety of incubated material after 0, 2,4,6 and 24 hours (Table 1) shows that there is never a statistically significant drop of the eosinophils in citrated or defibrinated blood incubated with steroids. Contrasting with Godlowski's (1952) and Muehrcke's (1952) experiments covering only 4 hours, our counts were extended to 24 hours incubation. Experiments with *Cortone*, *Hydrocortone*, cortisone aldehyde and dehydrocortisone acetate (Merck) Cortisone and compound F (Ciba), similarly display no significant drop in the counts during an incubation of 24 hours. Therefore the data of all experiments with these various substances are pooled in Table 1 under the two denominations, compounds E and F. The smears after 6–24 hours did not show any morphological changes within the eosinophil cells, nor did measurements according to the Price-Jones method reveal changes in size as reported by Padawer and Gordon (1952) in rats.

TABLE 1. EFFECT OF COMPOUNDS E AND F ON EOSINOPHILS AND LEUCOCYTES IN VITRO

		Number of experiments	Eosinophil and total leukocyte counts per cubic millimeter after:									
			0 hours		2 hours		4 hours		6 hours		24 hours	
			Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Control experiments without steroids												
Defibrinated blood alone	Eosinophils	12	1,227	486	1,131	425	1,116	418	1,129	437	1,128	553
	Leukocytes	12	6,367	703	5,808	686	5,342	602	5,183	629	4,490	411
Citrated blood alone	Eosinophils	12	3,233	1,277	3,456	1,481	3,241	1,281	3,368	1,427	1,958	382
	Leukocytes	12	10,150	1,496	9,968	1,486	9,484	1,351	9,638	1,630	9,473	1,445
Heparinized blood alone	Eosinophils	10	1,496	738	1,455	731	1,700	901	1,233	917	1,467	727
	Leukocytes	10	7,056	2,388	6,381	2,296	6,044	2,129	5,744	1,958	3,473	281
Experiments with Compounds E and F												
Defibrinated + Compound E	Eosinophils	9	1,987	1,138	1,884	1,100	1,781	1,138	1,497	830	1,555	892
	Leukocytes	9	4,660	400	4,585	530	4,250	473	3,922	453	3,874	593
Citrated blood + Compound E	Eosinophils	9	1,677	145	1,781	143	1,850	170	1,722	110	1,646	178
	Leukocytes	9	6,783	400	6,633	439	6,833	450	6,500	603	6,500	400
Defibrinated blood + Compound F	Eosinophils	9	852	293	675	231	514	164	516	131	493	94
	Leukocytes	9	4,888	546	3,825	511	3,386	440	2,925	348	3,271	375
Citrated blood + Compound F	Eosinophils	9	1,317	377	1,241	304	1,189	265	1,288	291	1,050	260
	Leukocytes	9	8,786	1,052	7,500	1,034	7,100	1,116	7,329	948	7,150	1,471

The statistical value used in this table is the standard error of the mean, $S.E. = \sqrt{\frac{\epsilon d^2}{N(N-1)}}$.

None of the variations in the mean total leukocyte and eosinophil counts after 0, 2, 4, 6 and 24 hours, with or without steroid addition, are significant according to the calculation, $M - M'$ significant when greater than $3 \times \sqrt{(S.E._M)^2 + (S.E._{M'})^2}$. This table is based on 350 duplicate total leukocyte and 350 duplicate eosinophil counts.

DISCUSSION

It may be seen from the data of experiments without steroids (Table 1), that the leukocyte and eosinophil counts in defibrinated and citrated blood remained as constant as in heparinized blood. Therefore no heparinized blood was used for the steroid experiments, thus precluding any methodical objections with regard to a possible inhibition by heparin of the glucocorticoid action of the eosinophil cell suspension (Muehrcke,1952; Godlowski, 1953.)

Blood samples from 36 human subjects with eosinophil counts ranging from 168 to 17,995 cells per cu. mm. of blood were used for pilot experiments, controls and steroid experiments. A total of 700 duplicate counts (350 total leukocyte and 350 eosinophil counts) were performed with the incubated blood. *No statistically significant drop of the counts in defibrinated or citrated blood incubated with or without glucocorticoids has been observed.* This evidence from experiments *in vitro* contrasting sharply with Godlowski's (1952 and 1953) and Muehrcke's (1952) reports, speaks against the contention that the eosinopenic effect *in vivo* of these substances is actually due to a direct eosinolytic mechanism.

SUMMARY

Conflicting results have been reported with regard to the effect of adrenocortical hormones on the eosinophils *in vitro*. This investigation is based on 700 duplicate counts of total leukocytes and eosinophils, in citrated and defibrinated blood samples, incubated for 24 hours with and without addition of various glucocorticoids. On the basis of the data presented it may be safely asserted, that these compounds do not have any eosinolytic properties *in vitro*.

A. F. Essellier, P. Jeanneret, E. Kopp and L. Morandi

Department of Medicine
University of Zurich
Medizinische Universitätsklinik
Zurich, Switzerland

REFERENCES

Baldridge, G. D., Kligman, A. M., Lipnik, M. J. and Pillsbury, D. M.: *Arch. Path.* **51:** 593. 1951.
Coste, F., Delbarre, F. and Basset, G.: *Rev. Rhum.* **19:** 327. 1952.
Essellier, A. F. and Wagner, K. F. *Acta Haemat.* **8:** 63. 1952a.
Essellier, A. F. and Wagner, K. F.: *Klin. Wchnschr.* **30:** 705. 1952b.
Essellier, A. F., Jeanneret, R. L. and Morandi, L.: *Blood* (in press).
Essellier, A. F., Marti, H. R. and Morandi, L.: *Acta Haemat.* (in press).
Godlowski, Z. Z.: *J. Endocrinol.* **8:** 102. 1952.
Godlowski, Z. Z.: E. S. Livingston Ltd., Edinburgh and London, 1953.
Muehrcke, R. L., Lewis, J. L., and Kark, R. M. *Science:* **115:** 377. 1952.
Padawer, J. and Gordon, A. S.: *Proc. Soc. Exper. Biol. & Med.* **80:** 581. 1952.
Panzenhagen, H. and Speirs, R. S.: *Blood* **8:** 536. 1953.
Quittner, H., Wald, N., Sussmann, L. N. and Antopol, W.: *Blood* **6:** 513. 1951.
Randolph, T. G.: *J. Lab. & Clin. Med.* **34:** 1669. 1949.

THE 1954 ANNUAL MEETING

The Thirty-sixth Annual Meeting of The Endocrine Society will be held in the Sir Francis Drake Hotel, San Francisco, California, Thursday, Friday, and Saturday, June 17–18–19, 1954.

Dr. Roberto F. Escamilla, 655 Sutter Street, San Francisco, California, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Sir Francis Drake Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 19, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

Hotel reservations can be made through the San Francisco Convention and Visitors' Bureau, Room #300, Civic Auditorium, San Francisco, California. They should be accompanied by the official AMA application form which appears during December of 1953 in JAMA.

Arrangements have been made for members of this society who do not belong to the AMA to be assigned rooms during the days of the meeting of the Endocrine Society. Applications from non-members of the AMA should specify dates of arrival and departure and be accompanied by a deposit of $10 per room.

THE LAURENTIAN HORMONE CONFERENCE

The Laurentian Hormone Conference of the AAAS will hold its 1954 annual meeting at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period September 5–10. Interested investigators and specialists in the hormone field may apply for attendance by writing to the Committee on Arrangements, 222 Maple Avenue, Shrewsbury, Massachusetts, for application blanks. Since accommodations at the hotel necessarily limit the attendance, only those persons submitting applications can be considered. The application blanks must be received by the Committee no later than May 24 in order to insure issuance of invitations as soon as possible thereafter.

The following program has been arranged:

I. Pituitary Hormones

Aspects of the biochemistry and physiology of neurohypophysial hormones.

Dr. H. B. van Dyke, Columbia University, College of Physicians and Surgeons.

The fate and metabolism of anterior pituitary hormones.

Dr. Martin Sonenberg, Sloan-Kettering Institute for Cancer Research.

The mechanism of stimulation of ACTH secretion.

Drs. Paul L. Munson and F. Norman Briggs, Harvard School of Dental Medicine.

Pharmacological alteration of adrenocortical function.

Drs. R. Hertz, W. W. Tullner, J. A. Schricker, F. G. Dhyse and L. F. Hallman, National Cancer Institute, Maryland.

II. Steroid Chemistry and Biochemistry

The use of microorganisms in the synthesis of steroid hormones and hormone analogues.

Drs. J. Fried, R. W. Thoma, D. Perlman, J. E. Herz and A. Borman, The Squibb Institute for Medical Research.

Some recent advances in the methods of isolation and the physiology and chemistry of electrocortin.

Drs. S. A. Simpson and J. F. Tait, Courtauld Institute of Biochemistry, Medical School, London.

III. Hormones and Abnormal growth

Experimental pituitary tumors—their induction mechanism and hormonal secretions.

Dr. Jacob Furth, Biology Division, Oak Ridge National Laboratory, Tennessee.

The endocrinology of neo-plastic growth.

Dr. Rulon W. Rawson, Sloan-Kettering Institute for Cancer Research.

IV. Hormones and Aging in Man

Some observations on hormones and phenomena of aging in man and woman.

Dr. Earl T. Engle, Columbia University, College of Physicians and Surgeons.

Studies of steroid metabolism in men and women of various ages.

Drs. G. Pincus, R. Dorfman, L. P. Romanoff, B. Rubin and E. Bloch, Worcester Foundation for Experimental Biology.

V. The Mechanism of Hormone Action

On the mechanism of action of insulin.

Drs. R. Levine and M. S. Goldstein, Michael Reese Hospital, Illinois.

Effects of ions and hormones on carbohydrate metabolism.

Drs. B. Hastings, C. T. Teng and A. E. Renold, Medical School, Harvard University.

VI. Hormone-Cardiovascular Interrelationships

Endocrine factors in experimental atherogenesis and blood pressure regulation.

Drs. L. Katz, J. Stamler and R. Pick, Michael Reese Hospital, Illinois.

Present status of the VEM-VDM (Ferritin) systems in relation to circulatory homeostasis.

Drs. E. Shorr, A. Mazur and S. Baez, Department of Medicine, Cornell University Medical College and the New York Hospital.

BOOK REVIEW

BASEDOW'S DISEASE. By H. Sattler. [English translation by G. W. and J. F. Marchand.] New York, Grune & Stratton, Inc., 1952, 605 pp.

It is not often one is startled on opening a medical or scientific book, but this volume provides the exception. In the first paragraph are listed the cardinal features of Basedow's disease or, as it is more widely termed in this country, "Graves' disease." Startling is the omission of one cardinal feature—hypermetabolism—an omission that is readily understood when it is realized that Sattler wrote his book in 1907, several years before it was generally known that elevation of the basal metabolic rate constitutes a cardinal feature of hyperthyroidism.

This book represents the lifetime experience of an astute observer of Basedow's disease whose work was done long before any specific therapy was available. The symptoms are described, discussed, arranged and dissected in great detail. The natural history of the disease is well illustrated. There is a short section on hyperthyroidism in children and in various animals.

This is not a book that will be read widely by the average medical student, teacher or practicing physician, but it is one that will hardly be passed over by those with a more than casual interest in the thyroid gland or in thyrotoxicosis. There is a wealth of clinical observations on the behavior of the disease, erratic and predictable, and on the variations and frequency of occurrence of the signs and symptoms. The description of the ophthalmic signs is especially good. The vast literature of two centuries (to 1907) is handled by resort to fine print, following an established pattern of the day. This makes for difficult reading, yet the meat of the book is in this fine print. Sattler's judgment, pertaining to certain concepts of the physiologic pathology of hyperthyroidism, examined now, in the light of the present-day knowledge, seems uniquely sound.

The natural history of Basedow's disease is not readily available today, either from personal experience or from teaching because, fortunately, the disease can be generally and fairly readily brought under control by any one of many therapeutic methods. The description of the course of thyrotoxicosis in its native, untreated state is the chief value of this book, for it is a picture that probably will never be painted again.

A. ALBERT